

nature

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Walking on two legs

THE regular two-way flow of scientists and technologists that has now been established between the People's Republic of China and the rest of the world has led to some fascinating discoveries about the way in which Chinese research is done and how it is managed. Now the Organisation for Economic Co-operation and Development (OECD) has produced a large-scale document, *Science and Technology in the People's Republic of China*, which attempts to produce an integrated viewpoint on the nature and evolution of China's science and technology system. It should be widely read, particularly by those just about to embark on a visit to China.

OECD's Committee for Scientific and Technological Policy has, of course, been analysing national science policies for many years, generally at the invitation of member countries who have, during the course of the analysis, staged a 'confrontation' with the OECD examiners. In this case, no Chinese were present at the seminar of thirty-odd science-policy experts and sinologists from a dozen countries out of which this report arose. But although it is the work of outside observers and it lacks some detailed information (which probably is all but inaccessible in China itself), the document does most clearly outline the dilemma of organising science and technology in an immense, partly developed nation with major demands both on the urban and rural sector and with a fluctuating but potent ideology in the driving seat.

China seems to live perpetually under the influence of two opposing forces; walking on two legs is how the Chinese describe their attempts to come to terms with these polarities. Elitism and egalitarianism; old and new; 'expertness' and 'redness'; theory and practice; ideology and technology; Mao and non-Mao. And even these poles are very mobile and slippery, as Simon Leys recently demonstrated in the *New York Review of Books* with a mischievous juxtaposition of some of the late Chairman's mutually-contradictory thoughts. Perhaps the fundamental question is whether China is governable at all—if not governed firmly, it is in danger of falling into anarchy and fragmentation. If governed firmly, it is in danger of being strangled by the very

growth of bureaucracy this tends to bring with it', was the way one participant in the seminar put it.

Small wonder then that China's recent scientific and technological past should be marked by grotesque ups and downs. During the Great Leap Forward and the two preceding years (1956–60), university enrolments more than doubled, and the science budget more than quadrupled. But the country wasn't yet equipped to use its graduates and in the next few years enrolments declined by about a third as 'realism' took over and smaller growth was accepted. But then came the next violent change, the Cultural Revolution of 1966. This time universities were all but annihilated for a period of a couple of years as the higher educational system came under attack on all fronts, including its alleged discrimination against workers and peasants. In the recovery period of the past few years students now go through at least two years of manual labour in rural areas before proceeding to higher education.

Science itself was relatively well protected at the beginning of the Cultural Revolution, by the '16-point Directive', which stated 'we must take particular care of scientists . . . who have made great contributions. Efforts should be made to transform slowly their world outlook and their style of work'. This protection did not last, as the pressures for egalitarianism and Chairman Mao's philosophy bit more deeply ('Intellectuals specialising in engineering are better, because they are in touch with reality. Pure scientists are worse, but are still better than those who specialise in the arts. Students of history, philosophy and economics have no concern with studying reality—they are the most ignorant').

The results of these upheavals are now widely known to a growing band of people. And amongst these there has been strong criticism of reduced academic standards, together with muted approval for the science-for-the-people approach. But in the past few years the tide seems again to have been moving slowly the other way, and probably with Mao's death (which came after this seminar had occurred) will move a little quicker. China may start again to walk even more evenly on two legs; this report will provide an excellent guide for the onlookers. □



Persepolis and Miami Beach

Alvin M. Weinberg, of the Institute for Energy Analysis at Oak Ridge, Tennessee, comments on the evils of energy from fossil fuels and nuclear fission.

MIAMI Beach and ancient Persepolis must have as little in common as any two places in the whole world. Yet in the past three months each was the site of a scientific meeting that in an unsuspected way impinged on each other and possibly on our whole future. At Miami Beach 80 climatologists, ecologists, oceanographers, energy soothsayers, and scientific administrators met to discuss carbon dioxide in the atmosphere. At Persepolis, 500 nuclear people met to discuss transfer of nuclear technology. I attended both meetings.

Miami Beach marked the first time that most of the United States workers on the CO₂ problem, plus a sprinkling of European experts, gathered to decide what might be done about the great uncontrolled experiment—the effect of the accumulation of CO₂ in the atmosphere on climate. The atmosphere contains about 700×10^9 tons of carbon as CO₂; its present concentration is 330 parts per million (ppm). The concentration has been increasing at about 1 ppm per year during the 20 years that Professor Keeling of the Scripps Institute of Oceanography has been monitoring it. This increase corresponds to about one-half the total CO₂ thrown into the atmosphere by the burning of fossil fuels.

How much of the increase in CO₂ really comes from burning fossil fuel, how much from clearing of the world's forests? The oceanographers were adamant: there was no room in the oceans to accommodate a contribution from forest clearing that was a significant fraction of the contribution from fossil fuel. The ecologists were less adamant: from what we know about forest cutting, we cannot say whether the biosphere is a net source or a net sink of CO₂. More experiments and observations are needed.

If one assumes the CO₂ increase is caused by the burning of fossil fuel, what is the likely ultimate level of atmospheric CO₂? Since removal of CO₂ to the deep ocean requires 500–1,000 years, the concentration of CO₂ would double in about 300 years, even at the present rate of burning fossil fuel. But if the world's use of fossil energy increases—say sixfold by 2050—then the CO₂ concentration might double in 75 years.

The climatologists, largely basing their conclusions on the Global Circulation Model of Manabe and Wetherald, came close to predicting that a doubling of CO₂ concentration would cause an unprecedented warming of the climate: about 2 °C average, 8–10 °C at the poles. Moreover, the usually invoked stabilisers, especially clouds, did not seem to change these estimates significantly.

What, if anything, can one do about CO₂? Schemes ranging from dumping the CO₂ directly into the deep ocean (a suggestion of C. Marchetti) to planting a trillion trees (F. Dyson) were described. But mostly there was an air of helplessness. If CO₂ is as big a problem as many now suspect, we simply may have to limit our use of coal and other fossil fuels or live with whatever consequences may arise. Prudence dictates that in any case we keep open the world's energy options—solar, fission, fusion, conservation.

Which brings me to Persepolis. The most important paper discussed there was not even on the agenda: it was President Carter's nuclear policy, which was announced on the eve of Persepolis, and which completely dominated the proceedings. How could it be otherwise? Persepolis aimed at facilitating transfer of nuclear technology; President Carter's policy, though domestic, was largely aimed at minimising proliferation and therefore at limiting the

transfer of certain nuclear technologies. No wonder most of Persepolis reacted as though mortally wounded.

Did Persepolis react too strongly? I think it is too early to say. After all, President Carter's proscription of plutonium recycling in light water reactors (LWRs) is simply a continuation of President Ford's policy. It goes beyond the Ford policy in deferring the Liquid Metal Fast Breeder Reactor (LMFBR). Now plutonium recycling in LWRs is only marginally economic in the US; and although it is unclear how the nuclear enterprise will permanently dispose of its wastes unless some reprocessing is allowed, I don't think it was the continued US proscription of plutonium recycling in LWRs that mainly bothered Persepolis.

Nor did President Carter's call for investigation of breeders other than the LMFBR bother me. I have long believed that the entire world locked itself into the LMFBR before it really investigated the alternatives. The real question to my mind is whether the President's policy represents a firm commitment to develop *some* breeder or was only the first step in soft-peddalling all breeder development.

The basic concern at Persepolis was that the new policy may betray an underlying inclination to have done with nuclear energy: let LWRs run their course, and because of the danger of proliferation, do nothing serious about the breeder. And if the breeder is not developed, nuclear energy will wither as our low-cost uranium is used up.

But what then of the necessity of keeping options open, a necessity made urgent at Miami Beach? At Miami Beach the alarm-sayers sense the end of fossil fuels because of CO₂. At Persepolis the alarm-sayers sense the end of nuclear energy, crucified on a cross called proliferation.

I would like to believe that both alarm-sayers are over-reacting: that President Carter's policy will really lead to the timely development of several breeders, and that CO₂ somehow isn't the Sword of Damocles pictured at Miami Beach. But these are hopes, not necessary realities.

To weigh proliferation against climatic change is surely beyond human wisdom. Yet there is an asymmetry in these catastrophes, assuming they really exist, that suggests a proper course. The CO₂ catastrophe, if it indeed exists, will occur even if the fossil fuel system works *properly*: it can hardly be avoided short of drastically reducing our use of fossil fuels. Proliferation, by contrast, could be, though not necessarily, a consequence of an *improperly* operating breeder system, that is, one in which technical and institutional barriers to proliferation have failed.

Proliferation stemming from breeders can in principle be forestalled by proper design, both technical and institutional; a CO₂ catastrophe (assuming it exists) hardly can be forestalled except by changing to a non-fossil energy system. It therefore seems clear to me that we turn away from serious development of breeders at great peril. I hope my concerns are without merit, and that the President's policy will lead to greater, not lesser, emphasis on the practical achievement of proliferation-resistant breeder systems. □

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Barnaby's

Plant a tree and save the world?

An aid to development?

How can science be made to help poor countries?

Alastair Hay looks at part of the UK and Commonwealth effort

TWO international conferences in the past month have tried to resolve the problems involved in dividing the spoils of the world between rich and poor. The first meeting, the Conference on International Economic Cooperation or 'north-south' dialogue in Paris, failed to live up to expectations. The second, the Commonwealth Heads of Government meeting in London last week, had less expected of it. Both have hitherto recognised one way or another a role for science and technology in improving the relationship between rich and poor countries. The difference is that the Commonwealth possesses the means to do something about it.

The final communique from the Paris meeting indicated that the ultimate goal sought by poor countries of a new international economic order was a long way off. No figure was put on a common fund to help stabilise raw material prices, a \$1,000-million aid package fell far below Third World needs, and a general moratorium on Third World debt repayments was refused; on energy the West's goal of discussions between oil producers and consumers was not achieved. But with the countries still talking to each other it was not an unmitigated failure.

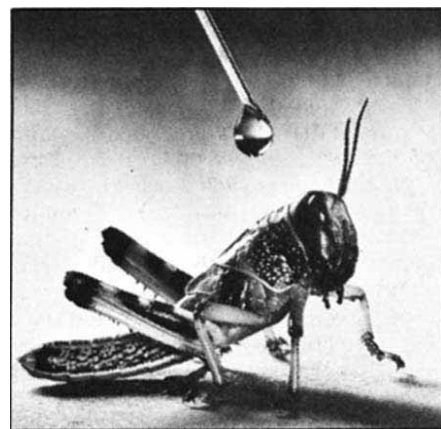
Some further progress came from the Commonwealth meeting. And in line with the view that the developed countries will need to provide more aid, the UK Ministry of Overseas Development (ODM) announced a higher contribution to the Commonwealth Fund for Technical Cooperation (CFTC). Through this multilateral development aid fund financed by all Commonwealth governments, the Commonwealth Secretariat supports activities undertaken by such organisations as the Commonwealth Science Council and provides technical assistance in scientific fields to Commonwealth governments. The Commonwealth Science Council (CSC) is the main agency for promoting scientific collaboration between Commonwealth countries, and the increase will have pleased the Commonwealth Secretary General, Shridath Ramphal. In a recent symposium in Brussels on science strategy for Europe he voiced the opinion that "structural change in the world's economic system is the pre-condition of real development". To achieve the new international economic order which the poor countries were seeking, he said, "an important, indeed an essential and integral element,

would be a new, enlightened and dynamic role for science and technology".

The Commonwealth's role in this sphere is not well known, not least because of its minuscule size. The CSC's aim is to increase Commonwealth countries' "national capability to use science and technology for economic and social development", an onerous task for an organisation with a 1976 budget figure of only £100,000 provided by its 26 member countries and £20,000 for project funding by the CFTC. The CSC had its origins in the informal scientific collaboration between Australia, Canada and Britain prior to and during the Second World War. A conference convened by the Royal Society in 1946 placed this collaboration on a more formal basis, and the Council expanded thereafter to incorporate other Commonwealth countries and had several name changes before finally adopting its current title in 1975. By 1972 with the formation of national science research councils in many developing countries most governments were able to define their development problems more specifically, and this helped the CSC to communicate more effectively with them and especially with the research councils.

The Commonwealth Geological Liaison Office, which publishes reports and a news letter containing information on minerals and geology of interest to Commonwealth countries, and the Permanent Commonwealth Collection of Microorganisms, which promotes the expansion of culture collections and their wider use within the Commonwealth, both function under the aegis of the CSC. Further Commonwealth science collaboration is promoted by the Commonwealth Agricultural Bureau and Institutes which disseminate research information in specialised fields of agricultural science. Two more technologically sophisticated science agencies of the Commonwealth are the Commonwealth Advisory Aeronautical Research Council and the Commonwealth Consultative Space Research Committee. And the Commonwealth Foundation exists to provide grants for specialist conferences, study and research visits, bursaries and lectures.

Like all organisations, the CSC has had its difficulties. A Working Group report in 1974 expressed the view that CSC's work should be expanded but with more coherence than was evident



Testing pesticides at the COPR

in the past; in this way it might get across to more scientists in the Commonwealth. Following this gentle upbraiding the CSC streamlined and concentrated its operations. Those of prime concern include standards for engineering and industrial products; industrial research and training; scientific communication and management; mineral exploitation; the technological infrastructure of rural communities; non-conventional energy resources; and preservation and storage of food.

It is a list to daunt many development scientists. The CSC's Executive Secretary since 1972, Gwyn Thomas, who combines his role with that of Scientific Adviser to the Commonwealth Secretary General, agrees that the CSC has a demanding brief, but stresses that its purpose is rather to act as a catalyst than to run specific development projects. The CSC is working towards coverage by organising regional conferences on one or other of these topics, with several interested countries participating. These meetings are held to define common problems and to assist with the formulation of a joint R&D strategy to solve them. It is also encouraging greater sharing between countries of facilities for industrial training. Member countries train technologists in the use of quality standards and there is a move to promote regional harmonisation of standards. As the CSC sees it, quality standards are essential if exports from developing countries are to be sold in highly competitive Western markets.

Crucial concern

The subject of food preservation and storage remains one of crucial concern to the CSC. In view of the Council's limited resources in this field, it is again restricted to exhortation and organisation of meetings. But two UK organisations echo CSC interest in this field and are able to participate actively in development programmes; as a result they provide much relevant

scientific data for food preservation in the developing countries. They are the Centre for Overseas Pest Research (COPR) and the Tropical Products Institute (TPI). Both are scientific units of the UK Ministry of Overseas Development, and as such assist the government of any developing country requesting help. There is a division between their respective roles. The COPR is concerned with pre-harvest food losses, the TPI with the post-harvest period.

The COPR is the newer of these two units. Formed in 1971 by the merger of four ministry units concerned with locust, tropical pesticide and termite research, it started life with its component units having over 60 years' experience in overseas pest control. With this background and the resources available to it—the unit's R&D budget for 1976 was £816,000—the Deputy Director, Tecwyn Jones, found it logical for COPR to "concentrate on pre-harvest loss control and public health fields involving insect vectors".

Jones's experience of pest and insect vector control in the tropics where harvests and health often cannot be guaranteed without pesticide use makes him conscious of the continuing need for chemical control. The objective is to guarantee farmers' crops in the developing world and to protect the health of the workers in the field. However, the COPR is fully conscious of the dangers and shortcomings of persistent insecticides and recognises the urgent need to search for alternative ways of control and use available insecticides more prudently. To this end it is promoting the use of ultra-low volume spraying of pesticides in the tropics. With this technology, sprays effective for the control of tsetse fly can be reduced from 1,000 grams of pesticide per hectare to as low as 6 grams per hectare. The COPR is presently investigating the use of synthetic pyrethroids as alternatives to such chlorinated hydrocarbons as endosulphan, DDT and dieldrin.

Control of insect pests in the future seems in the COPR's view to lie in the use of a combination of insect pheromones—to disrupt mating and reproduction habits—and viruses which are specific to certain pests. Natural parasites and predators may also have a role to play. A COPR programme to test pheromone/virus control has begun and field trials are to be conducted next year in the Mediterranean area, and in East and Central Africa.

On an international scale the COPR collaborates with UN agencies like FAO and WHO. It acts as a reference centre for FAO on locust migratory patterns, and research in the unit on plant resistance to specific insect pests could provide genetic information of

relevance to plant breeders. The unit serves as an international reference centre for WHO for the evaluation of new insecticides for disease vectors such as malaria mosquitoes and tsetse flies, and has an intensive programme to test new molluscicides on the bilharzia parasite.

Broader role

The larger and older of the ODM units is the TPI. With a history going back to 1894 it has amassed considerable expertise in the field of post-harvest processing, preservation, storage and marketing of tropical products. For most of its existence the institute ministered specifically to the needs of the Commonwealth. However, with its incorporation into ODM in 1965, it was able to participate in work directed to the developing world as a whole.

Malcolm Thain is a deputy director of the TPI and is involved in directing the institute's R&D work, which in 1976 had a budget of £1,058,000. He says that the 1975 ODM White Paper 'More help for the poorest' confirmed the TPI in its basic work and that it is generally acknowledged that for Third World crops there is insufficient information on where the losses actually occur in the chain from harvesting to the consumer.

Some of the TPI's research programme is therefore directed towards identifying the weak points in this chain. Inevitably, unfortunate side effects of the green revolution have been identified; varieties of maize especially bred for yield often prove to have poor storage qualities, for example. This has led many Third World farmers to revert to their own lower yielding but better storing varieties. The TPI is thus directing some of their efforts towards the problem of storage of high yield crops, genetic breeding for 'storage properties' as well as yield, and low cost technology for assisting farmers to both dry and store crops. For instance, cassava has a storage life of a few days before

decay sets in. TPI scientists have found that a simple earth clamp, similar to that used for storing potatoes, will preserve the crop for up to 3 months.

Other TPI research efforts are concerned with insect pests, again using pheromones as well as fumigation methods, and quality control trials on those foodstuffs of developing countries which have an export potential. One TPI department of increasing relevance to developing country needs is the Industrial Development Department (IDD) near Culham, responsible for most of the ODM research effort on appropriate technology.

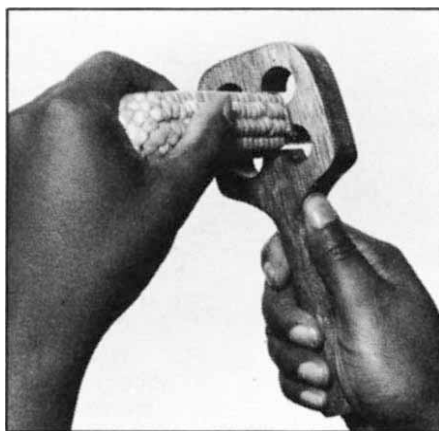
David Adair, the head of IDD, is one of those enthusiasts the field of appropriate technology seems to generate. He laments the fact that there is still insufficient grasp of the essential elegance and simplicity of some of the technologies the TPI is working on. Often because they lack the complicated gadgetry of much of today's sophisticated equipment, the items tend to be regarded with a certain air of condescension. However, Adair is not too concerned. Increasing orders for IDD's products is sufficient confirmation of the need for this technology in the developing world. A few of the unit's developments include a still for bay oil extraction which will run on leaves as fuel, a cashew nut processing plant, sunflower seed, cotton seed and groundnut decorticators, simple milling devices, and maize and coconut shredders. IDD places great emphasis on fuel conservation and devotes much attention to alternative sources of fuel like coconut charcoal and to non-conventional building materials.

Demand problem

Adair's problem—he receives more requests for assistance than he can hope to provide—is common to the TPI as a whole as well as to COPR and CSC. All three concerns publish extensive information about their activities and rely to a considerable extent on publicity to get their message across. But the resources required to meet the needs of the developing world are far greater than those possessed by these three organisations, or indeed by the international organisations or charities.

Development programmes in Third World countries embrace not only science and appropriate technology but sociology, ethology, medicine and economics as well. Integration of these disciplines into aid programmes is now an accepted practice, but personnel and resources are still too limited.

The solution is succinctly put by the Commonwealth Secretary General: "It is not lack of knowledge about what needs to be done, but the will and commitment to do it". □



Corn off the cob

ISRAEL

Exploiting an advantage

Nechemia Meyers learns of the likely prospects for science and technology under Israel's new government

"WE in the Likud realise that resource-poor Israel has a comparative economic advantage in only one sphere—science and technology—and so we will do everything we can to ensure that this advantage is exploited to the fullest possible extent". So says Professor Moshe Arens, the unofficial science spokesman for Israel's new Likud Government.

The Likud's concern with science and technology may stem in part from the fact that it has four men with a technological background in its 45-member Knesset delegation. Apart from Arens they are Professor Joseph Rom of the Haifa Technion's Department of Aeronautical Engineering, Mr Gustav Badian, Secretary-General of the Engineers' Union, and Mr Yitzhak Moday, who has a degree in chemical engineering but specialises in economic subjects.

The Likud does not favour the establishment of a separate Ministry of Science and Technology, however. This, it believes, would be counter-productive. "Instead of pigeonholing these subjects", declares Arens, "we must ensure that every single ministry

is concerned with science and technology, and particularly with exploiting Israel's own technological potential."

In the defence sphere, for example, Arens readily admits that in present circumstances Israel would still be dependent on certain American components, but argues that if there should be an American embargo on the supply of these components, they could be purchased elsewhere or produced at home. He points with pride to the fact that Israel's highly effective missiles, which he helped to develop, have brought in tens of millions of export dollars, and he is certain that many more high-technology civilian products could do the same.

"We are already selling a whole range of sophisticated items ranging from pharmaceuticals and laboratory chemicals to computerised whole body scanners for hospitals and computerised design systems for textiles", he says. "But much remains to be done. Take solar energy, a field in which we have pioneered. Even now Israel uses more solar energy per capita than any other country in the world, and she also sells some solar energy installations overseas. However, if our scientists are given proper encouragement, I'm convinced that they will be able to develop more efficient methods of exploiting solar energy".

According to press reports, Arens

may be put in charge of a ministry or an authority dealing with energy, water, building and other elements of what he calls the country's physical infrastructure. While refusing to comment on these reports, he does stress the Likud's view that the development of Israel's physical infrastructure must be more carefully planned, and its component parts utilised in a more rational manner.

Arens promises that Israel's R&D facilities, whether in agriculture, industry or some other sphere, will get all the support they require both from the Likud government and from private investors, whom the Likud believes it can encourage by creating a better climate for investment. However, the universities, which are almost entirely dependent on public funds, may be in for a tough time—after already suffering a drop of 30% in real income since 1973.

While Arens declines to estimate how much support the universities can expect, he does note that the Likud will have to slash the government budget if it is to reduce inflation (now running at over 40% a year). This means that at least for the next few years "short term targets will have to be given priority over longer-term targets". Being a faculty member himself, Arens doesn't lack sympathy for Israel's institutions of higher learning. But his presentation of the Likud's priorities gives them clear cause for concern. □

BRITAIN

Publish and be dammed

In the continuing debate over Britain's energy policy there is still no shortage of information being published. Chris Sherwell reports

WHILE final preparations were being made for the opening of the Windscale inquiry this week, one of the chief opponents of the controversial project was with impeccable timing publishing a new book seriously questioning Britain's commitment to an electro-nuclear future. It was not the only publication on the energy front, however. The UK Department of Energy (DEN) was busily announcing another set of findings regarding its strategy for developing alternative energy sources, this time for tidal power and particularly the Severn barrage. Four reports were published on this matter alone. The DEN also released a review, prepared at the end of last year, detailing the country's energy policy options.

The inquiry into British Nuclear Fuel Ltd's (BNFL) application to develop land at Windscale for an oxide fuel processing plant was due to open on Tuesday morning in nearby Whitehaven, Cumbria, before Mr Justice Parker, the inspector, and two assessors, Sir Edward Pochin and Professor Sir Frederick Warner. BNFL submitted an outline planning application to the local authorities in February. In accordance with his announcement last December, Mr Peter Shore, Secretary of State for the Environment, ordered the public inquiry to allow examination of health, safety, environmental and planning aspects.

The inquiry, which is expected to last some three months, is widely regarded as a critical test for the future of nuclear power in Britain. On it also hangs the fate of deals worth hundreds if not thousands of millions of pounds for reprocessing other countries' ir-

radiated oxide fuel. BNFL's statement of case, circulated in May, emphasises the conservation and waste control benefits of reprocessing and the economic advantages of taking on overseas business. Opponents, who include Friends of the Earth, the Conservation Society and others, have retained expensive legal counsel and sought to raise funds publicly.

The inquiry was expected to begin with brief opening statements on behalf of BNFL and the various opponents before proceeding later in the week to BNFL's detailed presentation. The basis for the sort of arguments the corporation will then face is contained in *The Fissile Society* by the FOE's energy specialist, Walt Patterson, and published last week*. With the inquiry almost certain to spill beyond the immediate issue of the reprocessing plant itself, the book is a useful articulate distillation of the case against a nuclear future that neatly ties together a historical perspective and the prevailing sense of urgency.

No such urgency is apparent in

connection with the long-standing and increasingly widely supported idea of a barrage across the Severn Estuary in the west country that would harness the energy from the tides. At a press conference to announce publication of results of three special studies relating to the barrage† and another more general report from the DEN§, Dr Walter Marshall, the department's chief scientist, could offer no date for a decision from the DEN's Advisory Council on Research and Development (ACORD) on the next step, a large-scale £1–2-million feasibility study.

This will not have encouraged people like Patterson, who argued strongly last week for a redistribution of Britain's energy R&D effort, and Arthur Palmer, the chairman of the House of Commons Select Committee on Science and Technology, from which a report on the barrage is due out in the coming months along with a separate report on other alternative energy sources. According to a spokesman for NEDECO, the Dutch engineering group which conducted one of the studies, the earliest that work could begin with a decision now is 1982, to allow for four years of preliminary study. Sixteen years later the project could be complete, at a cost variously estimated (depending on whether it is a 'single basin' or 'double basin' scheme) between £2,400 and £3,100 million at today's prices—excluding the turbines.

ACORD is now considering all the reports, which come 12 days after a one-day symposium on the subject in London. None of them examines the environmental effects of a barrage; that, says Marshall, would have proved both difficult and expensive. They were designed to consider, first, the influence of various barrage schemes on tidal levels and currents, and secondly, the technical feasibility of a barrage. The Hydraulics Research Station (HRS) study speaks cautiously of an increase in spring tidal ranges seaward of the barrages of about 1.4 m, but NEDECO speaks of a decrease of similar order. General changes in currents, however, would be small, but navigation, flooding and drainage would all be affected. On the positive side the Institute of Geological Sciences report finds the seabed at the barrage area uncovered by drift and with bedrock exposed. NEDECO's finding is the most positive: the complex project is technically feasible. The problems, therefore, are now economic and political. □

**The Fissile Society*, Walter C. Patterson, Pp. 117 (Earth Resources Research Ltd., London, 1977), £1.50.

†*Severn barrage study*, HRS, £2; *Severn tidal barrage scheme: pre-feasibility study of the closure of the estuary*, NEDECO, £4; *Geology of the Severn barrage area*, IGS, £1. (Obtainable from DEN, Millbank, London).

§*Tidal power barrages in the Severn Estuary*, Energy Paper No 23 (HMSO, £1.50). Also released, *Energy Policy Review*, Energy Paper No 22 (HMSO, £1.75).

SWEDEN

A developing nuclear policy?

Wendy Barnaby reports from Stockholm on recent developments concerning Sweden's nuclear programme

THE Swedish government is to give credit guarantees for the continued building of the Forsmark 3 nuclear reactor but not for Oskarshamn 3. In a classic case of a 'no-decision decision'—now the norm for the government's handling of the nuclear energy issue—the energy minister, Olof Johansson, said recently that about \$30 million would be guaranteed for Forsmark 3 while the reactor's owners agreed on the pace at which building should be continued. In a couple of months, when the negotiations were expected to be finished, the government would reconsider the question of more guarantees. He himself would not comment on the reactor's development, saying that he did not want to pre-empt the owners' negotiations.

The government gave no reason why guarantees to Oskarshamn had been refused, but a spokesman for the company said that there were two probable reasons. One is that, whereas the Forsmark reactors are 75% state-owned, Oskarshamn is a private company. In order to continue building their reactor without state financial guarantees, the management of Oskarshamn will probably have to approach ASEA-Atom—which is 50% state-owned—for help. The state could therefore gain some indirect control over the project.

The other reason is that the State Power Board, the largest owner of Forsmark's reactors, employs 2,000 workers who have been building Forsmark 2 and who would be unemployed if they could not now build Forsmark 3. The Oskarshamn practice, on the other hand, is to contract out its work. The government could therefore have been hoping to avoid visible unemployment.

Unemployment is very much on the minds of the industrial workers dependent on the reactors for their jobs. Six trade unions have sent a joint letter to the energy minister demanding an immediate, clear decision on the short and long term prospects for the construction of reactors. It is highly unlikely that they will get it. All they can hope for is clearer guidance in autumn 1978, when the government plans to present proposals for a new energy policy up to 1990.

The management of Oskarshamn is planning to go ahead with the construction of the third reactor, even

though it will be delayed now by up to two years. They reason that, if they can arrange their own finances, there is no reason why they should not in future be treated differently from Forsmark 3. The owners of each reactor will, under the law stating the conditions on which new reactors may be built, have to present the government with concrete proposals for the 'completely safe' storage of unprocessed fuel or of highly radio-active waste if the spent fuel is to be reprocessed; and reprocessing agreements will also have to be presented.

Although the Centre Party was elected to the government largely on its promises that it would stop nuclear power in Sweden, it has so far shown no particular hurry to do so. And if it is prepared to entertain the possibility of contributing to Forsmark 3, the Oskarshamn management evidently doubts that it will put in jeopardy the \$330 million-worth of orders placed last year with different sections of local industry for the components of Oskarshamn 3.

There is no more certainty about the reprocessing and storage of spent nuclear fuel than there is about the building of the reactors themselves. If the Ringhals 3 reactor produces any spent fuel before the end of 1979, it will be sent to La Hague in France for reprocessing. Otherwise, according to the technical director of the State Power Board, it will have to be stored without reprocessing. This solution may also have to be adopted for the Forsmark 1 reactor, which so far has no reprocessing agreement for the spent fuel it should begin to produce next year. The State Power Board envisages waste being stored in a central depot until 1995, by which time it evidently hopes that other solutions will have become available.

A second round of test drilling, designed to examine the quality of rock near Forsmark, is now being started by the Swedish Geological Survey on behalf of the companies building reactors. Preliminary results from the first tests, near Oskarshamn, were in general good, showing hard rock with low permeability—the sort of rock that would be suitable for storing nuclear waste. According to a spokesman for the nuclear companies, storage of processed, vitrified waste and the direct deposition of encapsulated fuel elements will be considered. The main geological reports are expected to be ready by 1 October this year, but supplementary reports will be issued during 1978. □

IN BRIEF

Orlov appeal

Almost 600 French physicists have signed a declaration in defence of Professor Yurii Orlov, founder of the unofficial Moscow 'Helsinki monitoring group'. This was announced this week at the close of the 'Orlov hearing', a mock trial staged at the Institute of Physics in London on the eve of the Belgrade review of the implementation of the Helsinki agreements.

Orlov was arrested on 10 February and is being held incommunicado in the Lefortovo prison in Moscow. He has not yet been formally charged, but it seems likely that when a charge is made it will be concerned with the activities of the monitoring group under the articles pertaining to anti-Soviet propaganda.

The case for the defence, presented in London by John Macdonald QC, analysed the 19 major documents on Soviet violations of the Helsinki agreements, maintaining that the content of the documents was true, and believed

by the members of the group to be true. "Witnesses for the defence" included the mathematician Leonid Plyushch, Vladimir Bukovskii, and Marina Voikhanskaya, a psychiatrist who refused to cooperate with the Soviet misuse of psychiatry for political ends.

Employment prospects

The total number of scientists and engineers employed by private industry in the United States dropped by 5% in the first half of the 1970s, according to a study released last week by the National Science Foundation. The first major survey of scientific employment since 1970, the study also indicated that there has been a major shift in employment patterns, with manufacturing industry reporting a 13% drop and non-manufacturing industries indicating an 11% increase in the number of scientists and engineers they employed between 1970 and 1975. The figures refer to all scientists and engineers working in private industry.

Those directly employed in research and development departments declined particularly sharply, from 372,000 in 1970, to 329,000 in 1975—a 12% drop. Though the survey covered a period of economic recession, the decline in the number of scientists and engineers employed by private industry was unexpectedly sharp. Recent indicators suggest, however, that the scientific job market may be picking up a little as the economy has improved.

Cost-of-living for NSF

Unmoved by protestations that academic science in the United States has been suffering from financial undernourishment, the House Appropriations Committee recently recommended that the National Science Foundation (NSF) should get no more than a cost-of-living increase for its support of basic research next year. The committee, which is the most powerful of the Congressional panels which handle NSF's budget, recommended that

RECENT reports that the United States is planning to build a radically new type of nuclear weapon, designed to kill people by releasing vast quantities of lethal neutrons while causing relatively little blast damage to buildings, may have been a bit misleading. Though details of the proposed weapons are shrouded in secrecy, a number of independent experts have told *Nature* that they are unlikely to differ significantly from the small tactical nuclear explosive already deployed in large numbers.

Funds to begin building the device, for use as a warhead on the Lance short range missile, are contained in budget proposals for the Energy Research and Development Administration (ERDA) which are now being considered by Congress. The first official public reference to the weapon came last March when General Alfred Starbird, Assistant ERDA Administrator for National Security, told a Congressional committee that it would be designed to "reduce the blast effect and get the kill radius you want through enhanced radiation".

All nuclear explosions give off energy in two forms: a blast and heat wave which causes physical destruction of buildings and other structures, and withering radiation in the form of gamma rays and fast neutrons (so-called prompt radiation) which can pass relatively easily through the atmosphere and penetrate solid material, including people.

In addition, radioactive debris from the explosive and from material sucked into the blast will later be deposited as potentially lethal fallout. It has long been known that the

Blasted heat



BACKGROUNDER

relative importance of these effects depends on a number of factors, including the size of the explosion, the design of the explosive, and the altitude at which it is detonated.

In general, the smaller the explosion, the greater is the relative output of prompt radiation. Design is particularly important: according to a book written two years ago by Theodore Taylor, a former weapons

designer, two explosives with the same explosive yield but with different designs could differ by at least a factor of 10 in their output of fast neutrons. In particular, thermonuclear fusion reactions, which take place in hydrogen bombs, are particularly efficient producers of fast neutrons.

Until recently, the chief goal of weapons designers was to produce high-yield explosives which would destroy targets through blast and heat. These are usually fission-fusion-fission devices in which a fission bomb is used to trigger a fusion reaction which in turn sets off a fission reaction in a blanket of uranium surrounding the device. More recently, design has shifted more toward so-called 'mini-nukes', weapons with small explosive yields which could be used with very accurate missiles to knock out small targets. Again, heat and blast would constitute the chief destructive force of the weapon.

The proposed new 'enhanced radiation' weapon is believed to be a modified mini-nuke, possibly a small fusion device, triggered by a low-yield fission explosion, without the surrounding blanket of uranium. The important point to note is that although the weapon is designed to give out relatively large amounts of neutrons, the heat and blast effects would still be substantial—according to some experts they would probably still account for the largest fraction of the weapon's energy output.

NSF should receive \$844 million next year. The administration had requested \$885 million. Virtually all the reduction would be taken from NSF's basic research programmes, leaving only enough for a 6.5% increase, about the same as the rate of inflation. The committee specifically stated in its report that it believes academic science has fared relatively well in the federal budget in recent years, and that a major boost in support is therefore unjustified. The Senate Appropriations Committee, which is often more generous towards NSF, has yet to act.

Catch quotas for whales

Last week, a meeting of the International Whaling Commission's (IWC) Scientific Committee was the focus for the latest exchanges in a long standing argument over how whale catch quotas are determined. A statement by the Committee, that no species of whale

is under threat from extinction, was challenged by Dr John Beddington of York University, one of Britain's delegates to the conference.

Dr Beddington claimed that the basis of the IWC's estimates of catch quotas, the 'maximum sustainable yield', is founded on a false premise. It states that a maximum harvest will be achieved if whale populations are reduced to 60% of their original size. Dr Beddington, however, believes that at this level whale populations will become unstable and may die out.

It is expected that protesters against whaling will be out in force during the Commission's main conference which begins on 20 June.

Tenerife allegation

The attempt to identify the victims of the world's greatest aircraft accident at Tenerife in March has involved "a travesty of justice and a major insult

to the field of forensic sciences in the United States", according to Bill Eckert writing in the latest issue of *Forensic Science* published by Elsevier Sequoia. He lists "classical mistakes" made by the Spanish authorities in the investigation. The bodies were removed from the wreckage without marking their location, and were embalmed and placed in coffins before identification was complete. They were examined by local public health and forensic authorities who had little knowledge of the procedure; no dentists were available. The bodies were also removed to another country and to a strictly military base using exclusively non-civilian experts. When other than the best available forensic experts are used, Eckert concludes, questions of a medicolegal nature may never be ascertained. This could affect "one of the greatest legal cases in the history of transportation accidents".

It has long been a standard agricultural practice to improve the soil by ploughing crop residues under ground. When I worked on a farm in the 1920s, a popular two-year rotation was to sow oats and sweet clover (*Melilotus alba*), cut the oats to leave a high stubble, let the clover grow to about eight inches the next spring, plough it under and plant maize. The benefits of such procedures were rediscovered by the late Mr Jerome Rodale, a New York City electrical contractor who turned to farming. He coined the names 'organic farming' and 'organic food' in 1942, and popularised them extensively thereafter. He said in 1964 that one MD in California "has cured four cancer cases by putting them on a 100% organic diet". Mr Rodale also used a machine called a 'Theramak' for taking electrical treatments to give himself "more electrical energy".

The definition 'organic' in terms of food production was given by Mr Rodale's son in 1972 at hearings before New York State Attorney General Louis Lefkowitz:

Organically-grown food is food grown without pesticides; grown without artificial fertilisers; grown in soil whose humus content is increased by the additions of organic matter; grown in soil whose mineral content is increased by the applications of natural mineral fertilisers; has not been treated with preservatives, hormones, antibiotics, etc.

The uncleanness of 'antibiotics' to the faithful followers of Rodale seems paradoxical in view of the fact that these organic compounds are produced by moulds. The term 'etc' makes the definition quite elastic. In the hearings, a New York State governmental investigator reported

that a man selling organic fish stated, verbally and on a placard, that it was caught in the pollution-free mineral-rich Atlantic Ocean. One trembles to think of the horrors of

What is organic?



THOMAS H. JUKES

inorganic fish, but, in any event, 'organic' food is a term coined by Rodale and maintained by his successors and heirs. Its popularity reflects the age-old struggle between reductionism and vitalism. Indeed, the legitimate scientific definition of 'organic' as referring to carbon compounds dates back to the concept that they are produced only by living organisms. This idea was annihilated in 1828 by Wöhler, who made urea from ammonia and carbon dioxide and announced he had synthesised urea "without a kidney, a bladder, or a dog". But vitalism lingers on in the

health food shops where fertilised eggs are sold at a 40% premium because of the "spark of life" imparted by the attentions of the rooster, and it is codified in Oregon, where the Department of Agriculture has issued standards for organic foods, containing "not more than 10% of the allowable residues of any pesticide or other synthetic or artificial substance as established by the FDA". Apparently small amounts of pesticides, like a touch of pregnancy, are permissible. Vitalism has now entered the pages of *Nature*, where we read (12 May) that 'Muck is magic'.

A taste panel at the University of Florida rated a large majority of 'regular' foods as superior to 'health' foods on the basis of "colour, flavour, texture, odour, and general appearance". However, 'organic' foods easily win the cash register battle for their sellers; they cost about 82% more than 'regular' foods in a USDA survey in 1972. Consumers can presumably take spiritual comfort in paying more for lower quality without the possibility of detecting an actual difference by analysis. However, a local case of Q fever is attributed to 'natural fertiliser' used in producing 'organic food'.

When my parents named me Thomas they had not heard the expression "I'm from Missouri", but I believe that they knew of the gospel according to St John, 20 v 24-25. In any case, a doubter I remain, despite Kenneth Mellanby's delight in seeing a headline "Ideas of organic farming being taken more seriously," and his evangelical call to nonbelievers in organic food to repent.

news and views

Biological macromolecules: outmoding the rigid view

from Barry Robson

X-RAY diffraction has been of immeasurable value in the analysis of the three-dimensional structure of biological macromolecules. However, the structures determined by X-ray diffraction of protein crystals in particular are inevitably biased towards a rigid picture because the molecules are 'frozen' by the constraint of crystal packing and periodicity. Crystallographers are therefore often accused of having too rigid a view of the protein as it actually exists in solution, although this is rather unfair since much useful information about flexibility in solution has been inferred indirectly from crystallographic studies. A recent study by McCammon, Gelin and Karplus in this issue of *Nature*, page 585, however, represents the most significant advance to date in quantifying the flexibility of a protein, and their technique can presumably be readily extended to other macromolecules.

In order to understand the possible implications of their work for the reinterpretation of the rigid crystallographic view, it is first useful to consider the theoretical work of investigators such as Cooper (*Proc. natn. Acad. Sci. U.S.A.* **73**, 2740; 1976). It is obvious that the structure in the crystal need not, in principle, be the same as the favoured structure in solution, although the evidence suggests that they are and implies the dominance of the intramolecular forces in the crystal. It is less obvious, if one forgets basic statistical reasoning, that the crystal structure need not be the same as the average structure in solution. Therefore Cooper has emphasised that, in the statistical mechanical distribution of protein conformations, the mode is not necessarily equivalent to the mean. In other words, although the most probable state of the molecule may be the compact globule observed by crystallographers, the properties

measured in solution may reflect the large number of possibilities for different, relatively open structures, each of which has a low probability but which collectively add up to a high probability. Therefore, although it is important to determine the degree of dynamic variability in a macromolecule, it is also important to establish whether or not this dynamic variability can draw the average conformation away from the most probable which is one hopes that observed in the crystal.

In order to resolve these uncertainties, McCammon *et al.* have carried out a computer simulation of the molecular dynamics in which the theoretical equations of motion of all the atoms in the molecule are solved simultaneously over a period of time, the history of the behaviour of the atoms being recorded for analysis. The system used was the small protein pancreatic trypsin inhibitor (PTI), the solvent being neglected except for four water molecules which are strongly bound in the interior. The structure obtained by X-ray crystallography was used as a starting point, and the atomic velocities were increased to yield an average kinetic temperature of 295 K. Analysis of the resulting trajectories revealed "a rich variety of motional phenomena that occur on the atomic level at ordinary temperatures". The internal motion of PTI was seen to be fluid-like and governed by collisions between neighbouring non-bonded atoms, rather than by rotational potentials governed by covalently bonded atoms. The average root mean square (r.m.s.) fluctuation of all atoms was 0.9 Å, although larger displacements of 3 Å or more occurred in some regions on the protein surface.

The value of 0.9 Å relates to the deviation from the average structure obtained by molecular dynamics. When the r.m.s. of all the atoms from the X-ray structure was considered, the deviation rose to 1.7 Å. This qualifies the relation between the dynamic

mean conformation and the statistical mechanical mode observed by X-ray crystallography, although if the answer is quantitatively well defined, it is not conceptually so clear cut. The authors state that the time-averaged structure is "near the X-ray structure but not identical with it".

The results are certainly not bad news for crystallographers: although the shift to the calculated time-averaged structure from the crystal structure might be to some extent an artefact of the models used for interatomic forces and of the neglect of solvent, a more correct treatment could undoubtedly bring about only better agreement. On the other hand, those who favour a flexible picture should also be reasonably happy as the molecule had quite clearly been sampling a series of neighbouring conformations whose average was not identical with the X-ray structure.

Not all of the PTI molecule was shown to be equally flexible. The largest fluctuations, apart from those due to the flexibility of side chains at the protein surface, came from the two ends of the backbone and residues 25–28 which loop out of the molecule. The latter showed particularly interesting behaviour, undergoing changes in shape superimposed on an oscillation with a period of 6 ps. On the other hand, regions of the chain which form into such characteristic local arrangements as α helix and β -pleated sheet have significantly smaller fluctuations than average. However, even these features showed oscillatory behaviour, the β -pleated sheet region participating in a low-frequency 'rippling motion'.

Despite the possible inaccuracy of the potential functions used, the neglect of individual hydrogen atoms, and the neglect of the surrounding solvent, these calculations show fairly clearly that PTI is able to wander through a large number of alternative conformations but is prevented by high energy barriers from wandering too far from the observed X-ray structure. This is

probably fortunate, because the neglect of the solvent is likely to be increasingly dangerous as conformations less and less like the X-ray structure are sampled.

Molecular dynamics and the nucleic acid controversy?

The potential for future application of molecular dynamics and related techniques seems enormous, and is likely to be as important in the field of nucleic acid structure. Of particular interest would be its application to the current heated debate concerning the 'zipper' model for DNA as an alternative to the classical double helix (Rodley *et al. Proc. natn. Acad. Sci. U.S.A.* **73**, 2959; 1976). X-ray diffraction of DNA fibres, as opposed to protein crystals, provides more ambiguous information with which the zipper model also seems to be in good agreement. While the double helix has the advantage of being, in some sense, aesthetically more pleasing, particularly because many other biological macromolecules form helical structures, it has to be remembered that nature does not work under an Arts Council grant. Any DNA structure, however bizarre, is likely to coexist in solution alongside the double helix if accurate energy calculations show it to be of comparable free energy, providing molecular dynamic calculations also show that the alternative structure is kinetically accessible. Although the zipper model appears to contain some bad interatomic clashes, there is a very real possibility that these can

be dynamically relaxed. This could be dynamically feasible; to make it kinetically feasible, the zipper and double helix arrangement would have to be able to coexist in the same strand with easy transitions from one arrangement to the other. While both criteria seem to be satisfied, the transition to a zipper structure can only occur by removal of right-handed twists from the ends of the DNA double helix. Although the current zipper model proposed alternate right and left-handed screw orientations of the chain of five nucleotides apiece, there seems no reason why multiples of five cannot be used, or sections employing different multiples of about five interspersed with classical double helix. Thus there is room for speculation that, while chromosomal DNA may be always double-helical, short sections of DNA in solution may show dynamic fluctuations to regions of zipper form, and greatly modify certain solution properties such as the kinetics of unwinding of short DNA *in vitro* (J. P. Day, personal communication). The important point is that all such conjectures can only be quantitatively justified, in the absence of conclusive experimental data, by theoretical tools related to molecular dynamics. The zipper-double-helix controversy, providing it is not debased by confining it only to the chromosomal DNA *in vivo*, is specifically a problem in molecular dynamics, and the machinery developed by workers like McCammon, Gelin and Karplus is available for resolving it. □

hepatitis B occur in drug addicts, homosexuals and prostitutes. Persons working in high endemic areas also suffer an increased risk of infection. Women of childbearing age in areas of the world where the carrier state in that group is excessive, are another segment of the population requiring immunisation in view of the increased risk of transmission of the infection to their offspring. Some authors also include military personnel. Consideration will also have to be given to people living in certain tropical and sub-tropical areas in poor socio-economic conditions and where hepatitis B infection is prevalent.

In view of the repeated failure to grow and passage hepatitis B virus (and indeed hepatitis A virus) serially in tissue culture, it has not been possible to develop a conventional vaccine against hepatitis. Attention has therefore been directed towards the use of other preparations for active immunisation. The foundations for such hepatitis B immunogens were laid by the demonstration of the relative efficacy of diluted serum containing hepatitis B virus heated to 98 °C for 1 minute in preventing or modifying the infection in susceptible volunteers (Krugman *et al. J. Amer. med. Ass.* **217**, 41; 1971). However, the use of heated whole serum or plasma is unlikely to be licensed for general use.

Hepatitis B small particle vaccines

Since hepatitis B surface antigen, the essentially protein coat of the virus, leads to the production of protective surface antibody as shown in experimental studies and serological surveys, the possibility of using purified 22-nm spherical surface antigen particles is attractive. Such experimental vaccines have been prepared from the plasma of apparently healthy carriers of this antigen. Human hepatitis B infection has been transmitted successfully to chimpanzees, and although the infection is mild, the biochemical, histological and serological responses in the chimpanzees are very similar to those in man. Sensitive laboratory tests are now available for the several markers of hepatitis B infection and these provide the means of monitoring vaccines. Limited numbers of susceptible chimpanzees have been shown to be protected by 22-nm particle vaccines that had been treated by heat or with formalin. Preliminary small-scale safety tests of such experimental subunit particle vaccines in volunteers are now in progress.

Hepatitis B vaccines

from Arie J. Zuckerman

VIRAL hepatitis has now been identified as a major public health problem occurring endemically in all parts of the world. In recent years, progress in the specific diagnosis of viral hepatitis has proceeded to such an extent that it has revealed a new type of hepatitis which is unrelated to hepatitis A (infectious or epidemic jaundice) or hepatitis B (referred to in the past as serum hepatitis). Precise virological criteria for this new form of hepatitis are not yet available.

The importance of hepatitis B is emphasised by its effects on every field of medical practice, the impact that it has on blood transfusion services and its association with chronic liver disease including cirrhosis and in some regions of the world with primary liver cancer. In addition, infection with hepatitis B virus, particularly in early life, may be

followed by the persistent carrier state. The carrier state may be associated with liver damage, and the world reservoir of carriers at present is estimated to number about 120 million.

Active immunisation against hepatitis B is needed for groups which are at greater risk of acquiring this infection. These groups include individuals requiring repeated transfusions of blood or blood products, prolonged in-patient treatment, patients who require frequent tissue penetration or need repeated access to the circulation, patients with natural or acquired immune deficiency and patients with malignant diseases. Viral hepatitis is an occupational hazard among health care personnel and the staff of institutions for the mentally retarded. High rates of infection with

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Last year, an experimental, partially purified subunit hepatitis B particle vaccine was administered to patients and staff of a haemodialysis unit. The study did not include a control group, but a marked difference in the incidence of hepatitis B infection was observed subsequently between the immunised group and a group of patients and staff who had not received the vaccine (Maupas *et al.* *Lancet*, **i**, 1367; 1976).

Although it is generally accepted that the viral subunit preparations when pure are free of nucleic acid and therefore non-infectious, the fact that the starting material for their preparations is human plasma obtained from persons who are persistent carriers of the surface antigen, which is a marker of hepatitis B virus, means that extreme caution must be exercised to ensure their freedom from all harmful contaminating material (World Health Organisation *Tech. Rep. Ser.*, No. 570; 1975). Some concern has also been expressed over the possible induction of harmful immunological reactions to host components, including pre-existing structures of the liver cells, which may be present in such particulate vaccine preparations (see Zuckerman *Nature* **255**, 104; 1975; *Lancet* **i**, 1396; 1976), but reactions of this type have not been observed with the highly purified experimental vaccines in the small number of chimpanzees and individuals immunised so far. The WHO Expert Committee on Viral Hepatitis has recently proposed certain minimum criteria and guidelines for the preparation of hepatitis B vaccines (World Health Organisation *Tech. Rep. Ser.*, No. 602; 1977).

Hepatitis B polypeptide vaccines

Attempts are also being made to prepare vaccines from the constituent polypeptides of the 22-nm spherical particles of the hepatitis B surface antigen. Vaccines prepared from such polypeptides would have an added margin of safety since they would be even less likely than the 22-nm particle subunit vaccines to contain infectious virus or contaminating host proteins that might lead to untoward autoimmune reactions in some individuals (see *Nature* **246**, 445; 1973). The potential use of antigenic polypeptides derived from hepatitis B surface antigen is under investigation in several laboratories. A number of these polypeptides are immunogenic and stimulate antibody formation in experimental animals to the group-specific antigenic determinant (Shih & Gerin *J. Immun.* **115**, 634; 1975) and subdeterminants of the surface antigen (Dreesman *et al.* *J. Virol.* **16**, 508; 1975; Gold *et al.* *J. Immun.* **117**, 1404; 1976). Certain of the separated polypeptides also induced a

cell-mediated immune response in experimental animals. In addition, a low molecular weight polypeptide component elicited a cell-mediated response to normal human serum, suggesting that at least one integral component of the surface antigen may contain or be intimately associated with an antigenic determinant related to a human serum protein (Cabral *et al.* *Infect. Immun.* **12**, 564; 1975).

The major problems with polypeptide vaccines at present are difficulties in obtaining sufficient quantities of the peptides in pure form and the relatively poor immunogenicity of the polypeptides when compared with the small particles.

Synthetic hepatitis B vaccines

An extension of the use of polypeptide immunogens is the isolation and structural identification of a small surface antigen peptide. This peptide could conceivably be synthesised, function as a hapten, and, when coupled to a suitable carrier may serve as a vaccine. A step in this direction has now been reported by Peterson and his colleagues (*Proc. natn. Acad. Sci. U.S.A.* **74**, 1530; 1977). In order to elucidate the structure of the surface antigen, it is essential, of course, to identify its proteins and other components, and much effort has been devoted to this work in many laboratories. Peterson *et al.* describe the identification of two major bands of polypeptides, with molecular weights of 22,000 and 28,000 respectively, and other bands with molecular weights of 16,000 and 40,000–90,000. The purified bands from the 22,000 and 28,000 molecular weight bands had essentially identical amino acid composition to each other and to the intact surface antigen. The most notable feature was the large amount of proline and half-cystine and a large percentage of hydrophobic residues. The amino-terminal and carboxy-terminal sequences of amino acids in these two major polypeptides, which accounted for 75% of the total protein, showed that hepatitis B surface antigen with determinants *adw* consists of a single major polypeptide chain or two homologous polypeptide chains that differ only in limited areas of their structure. The difference in the molecular weight of these two components is due to the carbohydrate moiety of the glycoprotein of the second band. Injection of the 22,000 molecular weight polypeptide emulsified in Freund's complete adjuvant elicited an antibody response in guinea pigs. Antibodies were produced to the group specific determinant and to one of the major subdeterminants of the surface antigen. The 28,000 molecular weight polypeptide, however, did not induce an antibody response and it is suggested

that the discrepancy in the immunogenicity of the glycosylated peptides reported by Dreesman *et al.* and Shih and Gerin (see above) could have been due to contamination of the two major polypeptides.

The momentum towards the production of unconventional hepatitis B vaccines is being maintained, and it would be imprudent to speculate whether successful cultivation of hepatitis B virus in tissue culture, which will lead to the development of ordinary vaccines, will be achieved before a synthetic vaccine appears on the horizon. □

DNA-protein interactions

from Michael Spencer

TWENTY-FOUR years after the unveiling of the Watson-Crick model for DNA it is still unclear how most of its associated proteins interact specifically with it. There are two basic problems: first, how particular DNA base sequences might be recognised by corresponding protein structures, and second whether the protein backbone could adopt a repeating secondary structure to match that of double-stranded DNA. The second of these questions is the subject of a recent paper by Church, Sussman and Kim (*Proc. natn. Acad. Sci. U.S.A.* **74**, 1458; 1977).

Their approach has a lineage going back to 1955, when the group led by Wilkins suggested (on X-ray diffraction evidence) that in nucleoprotamine there is an extended polypeptide chain following the narrow groove of the DNA B structure (*Nature* **175**, 834). This model was, however, relevant only to a limited class of nucleoproteins, because it relied for its stability on electrostatic interaction between DNA phosphate groups and oppositely charged arginine side-chains. Church *et al.* are interested in whether in other cases, such as the *lac* repressor-operator complex of *Escherichia coli* and the histone-DNA complex, there might be an alternative stabilising mechanism which would "set the stage for the specific recognition to take place".

The new work follows an earlier proposal by Carter and Kraut (*Proc. natn. Acad. Sci. U.S.A.* **71**, 283; 1974) that the interacting protein might adopt not a single-chain structure but a two-stranded β -chain helix. These authors

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had been struck by the observation of Chothia (*J. molec. Biol.* **75**, 295; 1973) that β -pleated sheets in proteins often adopt a right-hand twist, because this gives a lower free energy than the planar form. Carter and Kraut discovered that such a twist enabled a β -chain duplex to wind neatly round the outside of a nucleic acid helix. They decided, however, that the model was plausible only for the interaction of proteins with double-stranded RNA (not DNA) in the A form; it was then possible to form hydrogen bonds between the ribose 2'-hydroxyls and the polypeptide carbonyl oxygens.

Carter and Kraut were less concerned with present-day nucleoproteins than with a hypothetical ancestor formed in the prebiotic soup. Church *et al.*, more firmly rooted in modern times after the successes of their group with tRNA structure, persisted in trying to build models that would work for DNA in its biologically relevant B form. In order to match the two-fold symmetry of DNA they restricted themselves to antiparallel pairs of protein chains, but this still left them with two possibilities: the adjacent polynucleotide and polypeptide chains could run either antiparallel (type A) or parallel (type P). Polarities are defined here as running from 5' end to 3' end in a strand of DNA, and from NH₂ terminus to COOH terminus in the protein.

Both models were successfully built, using modern techniques of refinement, to incorporate stabilisation by hydrogen bonds from protein NH groups to the 3'-oxygens of the DNA backbone; this utilised all the free NH groups, half of the total being occupied with holding together the β -chain duplex. To obtain good stereochemistry it was necessary to modify the DNA structure, but in the case of type P the distortions were not serious; the atoms of the sugar-phosphate backbone were moved by no more than about half an Ångström unit. To justify the existence of type A models the authors have to invoke the possible existence of considerably distorted DNA structures, but the only evidence for these comes from some rather controversial work by Bram and Tougaard on fibre diffraction patterns (*Nature new Biol.* **239**, 128; 1972). At least some of the unusual patterns reported are now known to arise only with contaminated DNA (Selsing & Arnott *Nucleic Acids Res.* **3**, 2443; 1976).

Within the β -ribbon alternating side-chains project outwards in the models, so that basic residues could bond to DNA phosphates as in the 1955 proposal. The other alternating sidechains point inwards towards the narrow groove of the DNA, where they could be involved in recognition of the base

sequence. The authors hazard no guesses about how this might be achieved, but they helpfully provide atomic coordinates for aspiring model-builders to try their luck on the problem. Some possible interactions have already been described by Seeman, Rosenberg and Rich (*Proc. natn. Acad. Sci. U.S.A.* **73**, 804; 1976).

The weak feature of all this is, of course, the lack of direct evidence that any such structure exists *in vivo*, or *in vitro* either for that matter. Church *et al.* point out that sequence studies predict β -regions at the DNA-binding end of the *lac* repressor; they also quote evidence supporting the idea that binding occurs in the narrow groove of DNA. What is needed, however, is some crystallographic proof. If current rumours emanating from the eastern counties of England are correct, we may soon have a model of the nucleosome which will shed some light on the matter. A further possibility is that someone will be able to produce fibres of DNA complexed with a pure β -ribbon, rather than with whole proteins containing only limited regions of such a structure. If that happens it will be a cue for old-timers to blow the dust off their fibre-diffraction cameras, and to analyse a new and important structure. □

Sorting out the flu genes

from T. Barrett

THE influenza virus, apart from its importance as a human pathogen, is of great interest to virologists because of some unusual features of its molecular biology. Its surface antigenic determinants—the haemagglutinin and the neuraminidase—are constantly changing, leading to periodic influenza epidemics. When cells are coinfecting with different influenza strains recombinant strains are readily produced. This latter property led to the speculation that its genome was segmented, a fact recently confirmed by several laboratories. The genome consists of eight distinct single-stranded RNA segments (McGeoch *et al. Proc. natn. Acad. Sci. U.S.A.* **73**, 3045; 1976; Pons *Virology* **69**, 789; 1976; Ritchey *et al. J. Virol.* **18**, 738; 1976). The virus is negatively stranded, that is, the virion RNA (vRNA) must first be transcribed into complementary RNA (cRNA) before it can be translated into viral proteins.

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It was assumed that each vRNA segment represented a single gene since eight virus-coded proteins could be identified in infected cells. These proteins are the three P proteins, thought to have some function in virus transcription and replication; the surface antigens, haemagglutinin and neuraminidase; the matrix protein associated with the virus coat; the nucleoprotein associated with the RNA and finally a non-structural protein which performs some unknown function in infected cells but which is not found associated with the mature virus particle. Both the P proteins and the nucleoprotein are associated with the vRNA in the mature virus particle and can be isolated as a complex which is active in cRNA transcription (Rochovansky *Virology* **73**, 327, 1976).

Preliminary assignment of the gene-protein product relationship was based on the protein size, and the coding capacity of the vRNA segments (Inglish *et al. Virology* **74**, 489, 1976). However, there is room for considerable error in this approach and recent work has focused on getting more definite evidence for the gene-protein product relationship. Comparison of the electrophoretic mobilities of the vRNA segments and the virus-coded proteins of several influenza strains demonstrated significant strain differences, allowing the parental origin of both the vRNA segments and the virus-coded proteins to be determined in recombinants. Palese and his coworkers (for review see Palese *Cell* **10**, 1; 1977) have used these electrophoretic differences to map the gene functions of each RNA segment for two influenza strains—PR8 and HK. Analysis of recombinants which differ from either one of the parental types in one (or few) RNA segments allows one to deduce the gene product of this segment by assignment of the parental origin of the viral proteins. The results obtained with this technique confirmed that the gene order on polyacrylamide gels does not necessarily allow one to assign the protein coding function in order of size. For example in the two strains used in these studies, the fifth RNA segment (in order of electrophoretic mobility) of HK codes for the neuraminidase while in PR8 this segment codes for the nucleoprotein. In both strains the slowest moving RNA segment codes for the third largest protein. The technique, however, is time consuming in that suitable recombinants are required and to repeat the procedure on other influenza strains would require a great deal of effort. Recently a rather elegant and more rapid method of assigning gene functions to particular RNA segments has been developed by a group at Cambridge. Their approach to the problem exploits both the seg-

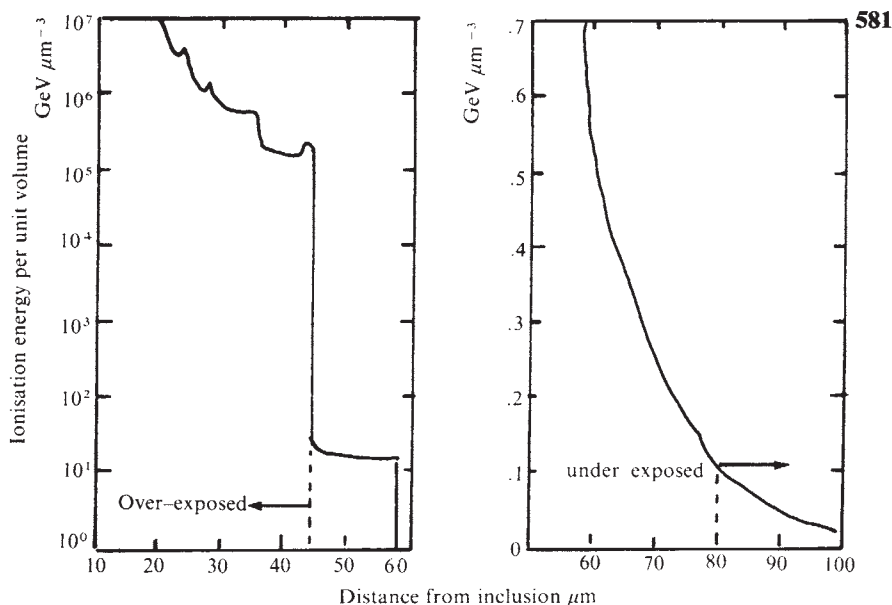
mented nature of the genome and its negative strandedness (Inglis, McGeoch & Mahy *Virology* **78**, 522; 1977). Individual mRNA functions can be selectively removed by hybridising total infected cell RNA with isolated individual vRNA segments. The individual vRNA segment will hybridise only with its complementary RNA, which acts as a message for a particular protein, and so make it unavailable for translation in a cell-free protein synthesising system. By analysing the protein products of the cell-free system, the protein for which a particular gene segment codes can be deduced by its reduction or total absence. This novel approach to the problem can be extended to any influenza strain and to other negative strand viruses. Another application which the authors suggest is the identification of gene coding functions of DNA restriction fragments. Since DNA-RNA hybrids are more stable than DNA-DNA hybrids in formamide the technique could be used to fish out mRNAs coded by particular DNA fragments. □

Giant haloes explained

from P. E. Hodgson

A POSSIBLE explanation has recently been proposed for the so-called giant haloes that are sometimes found in mica. The haloes are formed by alpha particles emitted from small inclusions of radioactive minerals inside the mica. These alpha particles ionise most strongly, and therefore cause the most damage, towards the ends of their paths, and so each inclusion is surrounded by a spherical shell of damaged mica, the radius of the shell corresponding approximately to the range of the alpha particles in the mica. In a thin sheet of mica, the region of damage appears as a circle, and the discoloured region of mica is called a pleochroic halo.

Many such haloes have been studied, and most of them have radii that correspond to the energies of the known alpha particles from radioactive minerals. However some haloes were found of much greater radii than these, indicating alpha particles of higher energy than any that are known to be emitted from radioactive minerals. These were called 'giant haloes', and it was conjectured that they might be due to the radioactive decay of superheavy nuclei with nuclear charges much greater than any known nuclei.



Cumulative alpha and proton ionisation as a function of distance from the centre of the halo. The arrows indicate the critical ionisations corresponding to the inner and outer edges of the halo.

Last year Gentry and colleagues irradiated with 6 MeV protons some inclusions of the mineral monazite that were associated with giant haloes, and found gamma rays were emitted that they associated with superheavy nuclei of charges 116 and 126 (*Phys. Rev. Lett.* **37**, 11; 1976; *Nature* **261**, 627; 1976). Later work, however, showed that these gamma rays can be attributed to the small amounts of cerium often found in this mineral, and extensive studies in many laboratories failed to find any evidence that there was any detectable amount of superheavy nuclei in other specimens of the mineral monazite (*Nature* **265**, 496; 1977).

This work effectively disposed of the claim that superheavy nuclei had been found, but there still remained the puzzle of the origin of the giant haloes. Did these indicate the presence of superheavy nuclei in such small amounts, or with such a short lifetime, that no trace of them remains in the present inclusions, or do they have some other explanation?

An alternative explanation of giant haloes has recently been proposed by Von Wimmersperg and Sellschop of the University of the Witwatersrand in South Africa (*Phys. Rev. Lett.* **38**, 886; 1977). They suggest that the giant haloes are formed when the mica contains some water. The alpha particles emitted from known radioactive minerals then collide with the protons in the water and knock them onwards. Since the rate of ionisation and hence the rate of energy loss is proportional to the square of the charge, protons have a much greater range than alpha particles of the same energy and so the knocked-on protons have on the average a much greater range than the alpha particles, and thus produce

haloes of greater radius.

Such a suggestion can be checked by calculating the radius of a halo that might be expected to be produced by a known radioactive mineral embedded in waterlogged mica. The cross sections for proton-alpha collisions are well known, and so it is quite straightforward to calculate the ionisation, and hence damage to the mica, that can be produced in this way. The calculated curve of ionisation as a function of radial distance from the inclusion is shown in the figure. The outer edge of the halo that would be produced by such a distribution can be estimated from the known threshold ionisation for alpha particles. The inner edge of the halo is determined by a reversal process that sets in above a certain level of ionisation; the mica becomes clear again, probably due to some annealing process. This is necessary for the observation of haloes by the proposed mechanism, since the ionisation goes on increasing towards the centre of the halo.

Assuming that the mass fraction of water in the inclusion is 0.3, Von Wimmersperg and Sellschop calculate that the inner and outer diameters of the haloes produced in this way are about 43 μm and 80 μm , compared with the measured values for giant haloes of 42 μm and 85 μm respectively. These results were obtained from reasonable assumptions relating to the proposed mechanism, without any attempt to adjust them to give an optimum fit. This certainly shows that the proposed mechanism is able to give haloes of approximately the required dimensions, but it still leaves some questions to be answered. It is not mentioned whether there is any evidence for such quantities of water in mica, though of course even if none were found it could always

be maintained that the water had been lost after the giant halo had been produced. It might also be thought that there should be some evidence of the halo produced by the alpha particles themselves, but as shown in the figure this would be removed by the annealing process at high levels of ionisation. □

Muonium hyperfine structure

from Peter Knight

THE muon is a curious particle. It behaves in all respects like a heavy electron, yet there seems to be no explanation of the large ratio of the muon mass to the electron mass. The atom muonium, consisting of a positive muon and an electron, is the simplest system available for precise investigation of the interactions between these so nearly identical particles. Everything we currently know about muonium is completely specified by Quantum Electrodynamics (QED) and the Bethe-Salpeter relativistic two-body equation. Its hyperfine structure is similar to that of hydrogen, but avoids problems of hadron structure so a precision measurement of the muonic hyperfine structure (hfs) checks leptonic QED to high accuracy and could perhaps indicate differences between muons and electrons. V. W. Hughes of Yale University has undertaken a systematic programme of precision experiments on muonium for the past 20 years, and with 11 co-authors has recently reported some greatly improved results on the ground state hfs (Casperson *et al. Phys. Rev. Lett.* **38**, 956; 1977).

Past work on the comparison of experiment with theory for muonium hfs has been limited by uncertainties in fundamental constants such as the fine structure constant α (this was most important), the velocity of light c and the Rydberg constant R_∞ . These constants are now known to higher accuracy. Experimental work was limited by the number of muonium atoms available. The basic principles underlying the latest experiment have remained more or less unchanged from earlier work, and the improvement in accuracy has been made possible by using the higher muon intensity produced by the Los Alamos 'meson factory' LAMPF. Muons are produced by the parity-violating pion decay $\pi^+ \rightarrow \mu^+ + \nu_\mu$ and are spin-polarised with the muon spin direction opposed to the linear momentum. Parity violation not only provides polarised

muons but also provides an elegant monitor of the subsequent evolution of the muon spin. The positive muon decays into a positron and two neutrinos with the positron emitted preferentially along the muon spin direction (and therefore in an opposite direction to the muon momentum). Atomic muonium is formed by charge capture from a krypton gas target at 1.7 and 5.3 atmospheres. The charge capture process does not alter the muonic spin directions so that spin polarised muonium is formed.

In zero magnetic field there are two hyperfine structure levels—a triplet state with total angular momentum $F=1$ and a singlet state with $F=0$ separated from the triplet by the hyperfine structure interval $\Delta\nu$, caused by the magnetic interaction between the spin magnetic moments of the electron and the muon. In the strong magnetic field (13.6 kG) used by Caspersen *et al.* the interaction of the magnetic moments with the external field is large compared with $\Delta\nu$. Figure 1 illustrates the magnetic-field dependence and state-labelling of the relevant $1^2S_{1/2}$ levels (the Breit-Rabi diagram). The relevant good quantum numbers are M_J , the magnetic quantum number for the electron, and M_μ the muon spin magnetic quantum number.

The external magnetic field is in the opposite direction to the muon spin, so $M_\mu = -\frac{1}{2}$ and the states $(\frac{1}{2}, -\frac{1}{2})$ and $(-\frac{1}{2}, -\frac{1}{2})$ labelled respectively 2 and 3 are created in equal proportions. If these muonium atoms are left to decay, the decay positrons are emitted preferentially 'backwards'—opposed both to the muon beam direction and the external field direction. Resonant microwave fields induce transitions at frequencies ν_{12} and ν_{34} between states $2 \rightarrow 1$ and $3 \rightarrow 4$; the muon spin direction changes and the decay positrons are then emitted preferentially in the 'forward' direction of the external field. In other words, resonant transitions are detected by the change in the angular distribution of the decay positrons. Sets of proportional counters and scintillation counters are placed on both sides of the target in a 13.6 kG external magnetic field produced by a precision solenoid.

Caspersen *et al.* have measured ν_{12} and ν_{34} . Measurement of both eliminates the need for a separate NMR measurement of the muon magnetic moment with its associated uncertainties. The combination $(\nu_{12} + \nu_{34})$ is used to determine $\Delta\nu$ and $(\nu_{34} - \nu_{12})$ to determine the muon magnetic moment μ_μ in terms of the proton magnetic moment μ_p . Their results are $\Delta\nu = 4463.302.35(52)$ kHz (three or four times more precise than earlier measurements) and $(\mu_\mu/\mu_p) = 3.183.3403(44)$. This latter can be used to obtain a new, most precise value for the muon-electron mass ratio $(m_\mu/m_e) = 206.76859(29)$. The difference between the theoretical value for $\Delta\nu$ (Brodsky &

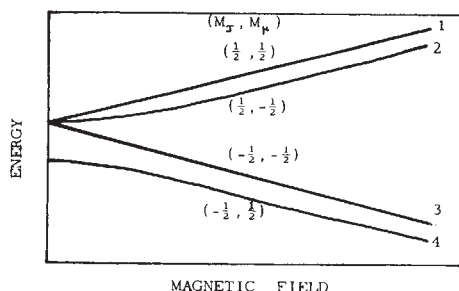


Fig. 1 Schematic diagram of Zeeman splitting of muonium hfs levels. At zero field, energy separation is $\Delta\nu$, the hfs interval.

Drell *A. Rev. nucl. Sci.* **20**, 147; 1970) and the new experimental value is 16.1 (6.6) kHz, 2.5 standard deviations of the combined error. The theoretical value is made up of several terms and the discrepancy may be due to an incomplete knowledge of the higher order radiative and recoil corrections to the bound state energy. These calculations are extremely complicated (for example Fulton & Repko *Phys. Rev.* **D14**, 1243; 1976). Weak interaction contributions such as neutral current effects are discounted by Caspersen *et al.* On the other hand, the measured density shifts of ν_{12} and ν_{34} indicate room for further experimental work. □

Biological sun-traps

from J. Coombs

A European Seminar on Biological Solar Energy Conversion Systems was held in Grenoble-Autrans on 9–12 May, 1977. It was organised by Professor D. O. Hall, Kings College, University of London and Dr P. M. Vignais, CNRS, Grenoble, and sponsored by a number of French research agencies.

THE idea of solar energy has risen like the phoenix from the ashes of the world's fossil fuel reserves. The number of publications, reports and research proposals grows steadily, but how realistic are they? Some 300 scientists assembled at Autrans, under overcast skies and persistent drizzle to review the present state of the application of biological solar energy conversion.

The basic tenets remained unquestioned—that solar energy represents the only significant safe, renewable, non-polluting future source

of energy and chemical feed-stocks. Unlike the advocates of solar energy technology however, biologists suffer from the fact that much of the relevant work is not new, but really photosynthesis, farming and fermentation in another guise. Problems more often relate to application of existing knowledge than to innovation. This is reflected in proposals for the solar energy programme of the European Economic Community in which less than 15% of the expected budget is going to biological projects. Exceptions to this generality, where more basic research is needed, include the possible chemical control of photorespiration, dinitrogen fixation, microbial utilisation of highly lignified cellulose and photobiological production of hydrogen gas.

Reviews of current status of knowledge of photosynthesis (Y. de Kouchkovsky, CNRS, France) and crop systems (P. Chartier, INRA, France) served to define energy flows and metabolic pathways which could give a potential net harvest of about 6% of the 3.8×10^{24} J yr⁻¹ received from the Sun. This contrasts markedly with the total world estimate of 3×10^{21} J yr⁻¹ trapped in photosynthetic products. The energy recovered in useful agricultural produce is several orders of magnitude lower, and there is obvious scope for improvement. However, it was suggested (R. W. F. Hardy, E. I. DuPont de Nemours & Co., USA) that, in the short-term at least, attention to environmental factors, improved varieties, fertilisers and crop protection, coupled with changes in farming objectives—such as a reversal in the decline of high protein grain legume cultivation compared with low protein cereal grain production—will produce the greatest benefits.

With an increasing world population food production must remain the chief aim of agriculture. Plant products to be used for fuel or chemicals must therefore be derived either from agricultural wastes or from new cultural systems—preferably ones which do not encroach on existing agricultural land. Several approaches to this problem were presented.

It is clear that all agricultural systems produce waste, but fortunately not all on the scale described for salad production in France, where studies by J. M. Legarg and A. Buatois, (Université de Lyon) indicated that in some circumstances less than 10% of the produce is eaten. In general, however, reliable estimates of wastes are not available. Detailed surveys carried out by a team from Stanford Research Institute, USA (J. A. Alich *et al.*) in Sutter County, California indicated that (perhaps not surprisingly) manures

tended to be overestimated and forestry residues underestimated by as much as 30%. In this particular county the 1.6 million tons of low moisture field residues located could provide 2,050 million kilowatt hours of electricity or 13,300 million cubic feet of synthetic natural gas each year. In both cases these figures exceed the present consumption of energy in the given form. This conclusion does not take account of the fact that the bulk of such wastes may be profitably returned to the soil, and that collection may be difficult and costly. In general mill wood and bark residues seem to be most suitable for use as a fuel for process steam or electric power generation. Another important form of waste utilisation is treating straw with ammonia or alkali, a process which doubles the fodder energy value whilst considerably reducing the bulk (M. Israelsen, Bioteknisk Institut, Denmark).

Studies of the technical and economic feasibility of obtaining energy from crops in Australia (H. D. W. Saddler, Australian National University, Canberra), Silvicultural Biomass Plantations (R. E. Inman, The MITRE Corp., Virginia), and marine culture of giant kelp (H. A. Wilcox, Naval Underseas Center, San Diego, California) remain as paper studies, as a result of problems of economics, energy balance and technology. It was therefore refreshing to hear of the commercial production of *Spirulina* (H. Durand-Chastel, Sosa Texcoco, Mexico) and the potential for glycerol production from the alga *Dunaliella* (A. Ben-Amotz, Haifa, Israel), although these were small scale in comparison with the algal ponds used for sewage and waste disposal in the US as described by J. R. Benemann (University of California).

In spite of the lack of large-scale applications for immediate use, optimism was raised by the quality of much of the basic work reported. Here there was a definite emphasis on biological nitrogen fixation. R. A. Dixon (University of Sussex) indicated the possibilities of manipulating genetic information associated with symbiotic organisms, although the possibility of transferring the ability to fix nitrogen to non-legumes remains simply a speculation. The contribution of non-symbiotic nitrogen fixation and loose associations of bacteria with plants remains difficult to estimate. Using a field assay J. Balandreau and Y. Dommergues (CNRS, Nancy) found less than 10 kg N ha⁻¹ yr⁻¹ being fixed in both African savannah and around maize plants. The elegant studies of K. E. Eriksson (Swedish Forest Products Research Laboratory, Stockholm) on enzyme mechanisms in-

involved in fungal degradation of lignocellulosic materials could be particularly important since at present no practical process exists for the production of glucose syrups from cellulose, the most abundant carbohydrate.

Hydrolysis, pyrolysis and fermentation of plant products give intermediates which can be converted to most of our present chemical feed-stocks using existing technology (P. Dupuy, INRA, Dijon). The impetus for implementing such technology will come as oil production peaks between 1980 and 1990. Production of ethanol as a fuel by fermentation of raw cane juice is already being investigated in Brazil. More exciting is the possibility of producing hydrogen gas by short-circuiting the photosynthetic process, using reconstituted systems produced by coupling of chloroplasts to hydrogenase as described by D. O. Hall. But both this system and synthetic photo-electric-chemical cells are a long way from practical realisation (A. San Pietro, Indiana).

Although nothing startling emerged from the meeting, a considerable change in the general attitude was noticeable. Proposals are becoming more rational and their limitations are being assessed more critically. The immediate effect may be to reduce waste of both energy and materials. In the longer term it is clear that new avenues of thought are being opened—discussions started during the conference continued unabated as the coaches returned us to the airports—and at last the Sun was shining. □

Magnetism in amorphous alloys

from F. E. Luborsky

An informal meeting on Magnetism in Amorphous Alloys was held on 6 May, 1977 at the University of Sussex, under the sponsorship of the Materials Science Division of the School of Engineering and Applied Sciences. It was organised by Dr A. W. Simpson (University of Sussex) and Professor E. P. Wohlfarth (Imperial College). Participants were invited from the chief European laboratories active in this field.

THERE has been intense research during the past few years on the ferromagnetic behaviour of certain alloys, especially those based on iron and nickel, which can be produced in the form of glassy

tapes by rapidly quenching from the melt. Several of these alloys have attractively high permeabilities and low magnetic losses and there is widespread interest in their possible applications as laminations in transformers and also in smaller electronic devices and in 'bubble memories'. Apart from this technological potential, the purely scientific questions raised by 'amorphous magnetism' have also attracted much attention.

What do we know about the origin of the magnetic properties of metallic alloys that are amorphous? What are the significant unanswered questions related to amorphous magnetism? What is the potential for application of these new transition metal amorphous alloys? These were some of the questions which the Sussex meeting hoped to answer.

In the only formal lecture of the day E. P. Wohlfarth introduced the subject, discussing why interest has arisen in the magnetic properties of the metal-metalloid amorphous alloys, which show soft magnetic properties with applications in transformers, inductors and magnetic sensors. He also discussed what the magnetic properties can tell us about the structure; the reawakened interest in itinerant compared with localised models of magnetism; and finally the origin of the magnetic properties of technological interest.

During the lecture and the discussions which took up the rest of the day many interesting ideas emerged, a few of which are summarised here.

It now seems clear that the non-crystallinity influences the primary magnetic properties in relatively small ways as the result of the variance, or distribution, in the exchange integral. This may be observed directly, for example, in the distribution of hyperfine fields obtained from Mössbauer spectroscopy. However the critical indices of crystalline and amorphous alloys are essentially the same. The relatively large differences in saturation magnetisation and Curie temperature between crystalline and amorphous alloys occurs because of the presence of the metalloid, which is necessary to stabilise the amorphous phase, and not because of the lack of crystalline order.

It was pointed out that very little is known about the effect of preparation variables (quench rate, melt temperature, annealing) on the variance of the exchange integral. The itinerant electron model, as for conventional crystalline alloys, provides a better description of the primary magnetic properties of amorphous alloys than the localised model. There is a smoothing out of the density of states curve for amorphous alloys but since the Fermi level is near the middle of the band, nothing much

will happen to the magnetic moment.

Factors which contribute towards making good soft magnetic materials include primary properties such as moment and Curie temperature, as well as the secondary properties such as the various anisotropies present, magnetostriction and inhomogeneities. The effect of preparation conditions and subsequent mechanical treatment and heat treatment on these secondary properties is particularly important. There are basically three sources of anisotropy: stress-magnetostriction, dipole effects arising for example from inhomogeneities in M_s or from directional atomic pairing, and spin-orbit coupling. The stress-magnetostriction interaction anisotropy is well understood from the many years of work with conventional crystalline alloys. For the amorphous alloys, the stress is understood to develop during the rapid solidification or during subsequent handling, but a fundamental description of magnetostriction and the effect of the non-crystalline structure on magnetostriction are both lacking. The presence of directional atomic order

arising from transition metal atom pairs and metalloid interstitial ordering has been shown to be the dominant source of anisotropy after annealing to minimise or remove the stress-induced magnetostrictive anisotropy. Thus the secondary magnetic properties are controlled by the same kinds of anisotropy as in crystalline alloys, but the magnetocrystalline anisotropy is minimal in a wide range of transition metal-metalloid amorphous compositions. This results in a wide range of compositions, with a range of magnetisations and other properties, of significant technological potential.

Wohlfarth summarised prospects in the near future for transition metal-metalloid amorphous alloys by suggesting that in the next few years there will be more theoretical work on the itinerant and localised models, there will be more relevant measurements on a variety of alloys, and new useful soft magnetic alloys will be developed.

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A hundred years ago

THE *Turkestan Gazette* gives the latest news from M. Prshevalsky, dated from Lob Nor, February 22. After having reached this lake by the valley of the Lower Tarim, M. Prshevalsky advanced 130 miles east of the lake. The survey and the astronomical measurements of latitudes and longitudes he has made give a totally new aspect to the map of the country. The population on the banks of the Tarim and around the Lob Nor is very sparse; the people speak almost the same language as that of Eastern Turkestan. The flora and fauna of the locality are very poor; some vegetation is found only in the Tarim valley, the neighbourhood being a true desert. During February and March M. Prshevalsky was to stay in the Lower Tarim, during May at Yuldus, and during June at Kunghes. About the beginning of July he proposes to return to Kuldsha to begin in August his journey to the Tibet.

A RUSSIAN work, by M. Bogolubsky, on Gold and Gold Mining in Russia, is worthy of notice. It contains very interesting information upon that industry in Russia and Siberia. We observe that the area of gold mines occupies in the Russian empire about

2,100,000 square miles, and now yields yearly about 80,000 lbs of gold, in value upwards of 3,000,000 l sterling. The total amount of gold produced in Russia since 1752 has been upwards of 2,500,000 lbs.

MUSCULAR contraction, it is known, is always accompanied with electric phenomena; the difference of electric potential between two points of a muscle, undergoes a diminution, which according to Bernstein, proceeds by about 1/100 of a second the contraction of the muscle. This *electric variation* has been observed in various muscles and in particular on the heart (by Du Bois Reymond and Kühne), and recently M. Marey has represented it graphically by photographing the indications of a Lippman capillary electrometer. We learn from the *Journal de Physique*, that M. De la Roche has tried the experiment on the heart of a living man. Two points of the epidermis of the chest were connected with the poles of a capillary electrometer, by means of electrodes, formed each of a bar of amalgamated zinc with a plug of muslin at its lower end saturated with sulphate of zinc. Held with insulating handles, the bars were applied, one with its plug opposite the point of the heart, under the left nipple, and the other to another point of the chest. The mercurial column was then seen to execute a series of very distinct periodical pulsations synchronous with the pulse; each pulsation even marked the double movement of the heart (of the auricles and ventricles). The amplitude corresponded to about 1/1000 Daniell.

From *Nature* 16, 14 June, 131, 133; 1877.

articles

Dynamics of folded proteins

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The dynamics of a folded globular protein (bovine pancreatic trypsin inhibitor) have been studied by solving the equations of motion for the atoms with an empirical potential energy function. The results provide the magnitude, correlations and decay of fluctuations about the average structure. These suggest that the protein interior is fluid-like in that the local atom motions have a diffusional character.

RESULTS of X-ray crystallography provide a picture of a globular protein in its native conformation as a well defined, densely-packed structure. Other experimental data¹⁻¹⁰ and theoretical considerations¹¹⁻¹³ indicate that there is considerable local motion inside a protein at ordinary temperatures. Moreover, the structural data themselves show that significant residue or subunit displacements have an important role in the activity of proteins (for example, enzyme catalysis¹⁴, haemoglobin cooperativity¹⁵, immunoglobulin action¹⁶). To obtain a more complete understanding of proteins, it is essential to have a detailed knowledge of their dynamics. In spite of the considerable effort directed toward protein folding¹⁷, very little has been done to investigate the motions of a protein in the neighbourhood of its equilibrium configuration. For certain cases in which the displacements along a suitably chosen coordinate can be isolated (for example, aromatic side chain rotations in the pancreatic trypsin inhibitor¹⁸, the opening and closing of the active site cleft in lysozyme¹²), it has been demonstrated that empirical energy functions can be used to analyse the motion involved. Here we undertake a more general examination of the internal dynamics of a folded globular protein. The approach used is of the molecular dynamics type¹⁹. In such a study the classical equations of motion for all the atoms of an assembly are solved simultaneously for a suitable time period and detailed information is extracted by analysing the resulting atomic trajectories. The full interatomic potential can be used to obtain the forces on the atoms, so that the method is applicable even when the system is highly anharmonic. The molecular dynamics approach has been very successful in revealing structural and dynamical characteristics of fluids¹⁹. As we demonstrate in what follows, molecular dynamics can have a corresponding role for the internal motions of proteins.

Bovine pancreatic trypsin inhibitor (PTI) (see Fig. 1a) was selected for study because of its small size (58 amino acid residues), high stability and accurately determined X-ray structure^{20,21}. Four water molecules, which are strongly bound in the interior, were included in the dynamical simulation. The potential energy was represented by an empirical function¹⁸ composed of a sum of terms associated with bond lengths, bond angles, dihedral angles, hydrogen bonds, and non-bonded (van der Waals and electrostatic) interactions. Hydrogen atoms are not explicitly considered, but are combined with the heavy atoms to which they are bonded by a suitable adjustment of atomic parameters. This use of 'extended atoms' reduces the

number of interactions which must be calculated and also permits larger steps in the trajectory calculation since the high frequency hydrogen vibrations have been eliminated. Integration of the equations of motion was performed by means of the Gear algorithm²² with time steps of 9.78×10^{-16} s. X-ray coordinates²¹ were used for the initial positions and the initial velocities were set equal to zero. After 100 equilibration steps, the stresses in the initial structure had partly relaxed and the system had an internal kinetic energy corresponding to a temperature of 140 K. At this point, all velocities were multiplied by a factor of 1.5 and 250 more equilibration steps were taken. The added kinetic energy ($250.6 \text{ kcal mol}^{-1}$) partitioned itself between kinetic and potential terms during this interval, and an average kinetic temperature of 285 K was reached. The actual simulation consisted of 9,000 additional steps, corresponding to 8.8 ps. Some equilibration of the bond lengths, bond angles and electrostatic interactions continues during the first 3 ps of the simulation, producing a small rise in temperature. Over the whole simulation, the average temperature is 295 K and the total energy is well conserved, changing by only $0.7 \text{ kcal mol}^{-1}$.

In what follows we first describe results concerned with the relation between the time-averaged and X-ray structures and with the magnitudes of structural fluctuations. We then examine the details of the dynamics, the correlation and damping of fluctuations, and some examples of the larger changes that occur. Of primary importance are the conclusions reached concerning the fluid-like nature of the internal motions, which will clearly have to be considered in developing dynamical models for biological processes.

Time-averaged structure and fluctuations

The time-averaged structure obtained in the dynamics run is near the X-ray structure but not identical with it; the root mean square (r.m.s.) deviation of the α carbons is 1.2 Å and that for all the atoms is 1.7 Å. The largest deviations come from the two ends of the molecule, the external loop (residues 25-28) and exposed sidechains. The two parts of the β sheet (residues 18-24, 29-35) and the α helix (residues 47-56) have significantly smaller deviations.

Use of the X-ray structure as the starting configuration corresponds to $0.4 \text{ kcal mol}^{-1}$ of kinetic energy per atom after equilibration (140 K); for the main dynamics run (295 K), the mean kinetic energy per atom is about $0.9 \text{ kcal mol}^{-1}$. Although there is no tendency to unfold, the dynamic development with this kinetic energy permits the molecule to make small rearrangements throughout; a picture of the structure after 3.2 ps is shown in Fig. 1b. Apparently the PTI molecule samples a series of neighbouring conformations, the average of which is not identical with the X-ray structure. It is not clear how much of the difference is a real effect (in the sense that the structure in the crystal is constrained) and how much is due to inaccuracies in the potential function and the absence of solvent. In lysozyme, comparison of the X-ray structures for the triclinic and tetragonal crystal forms^{23,24} shows an r.m.s.

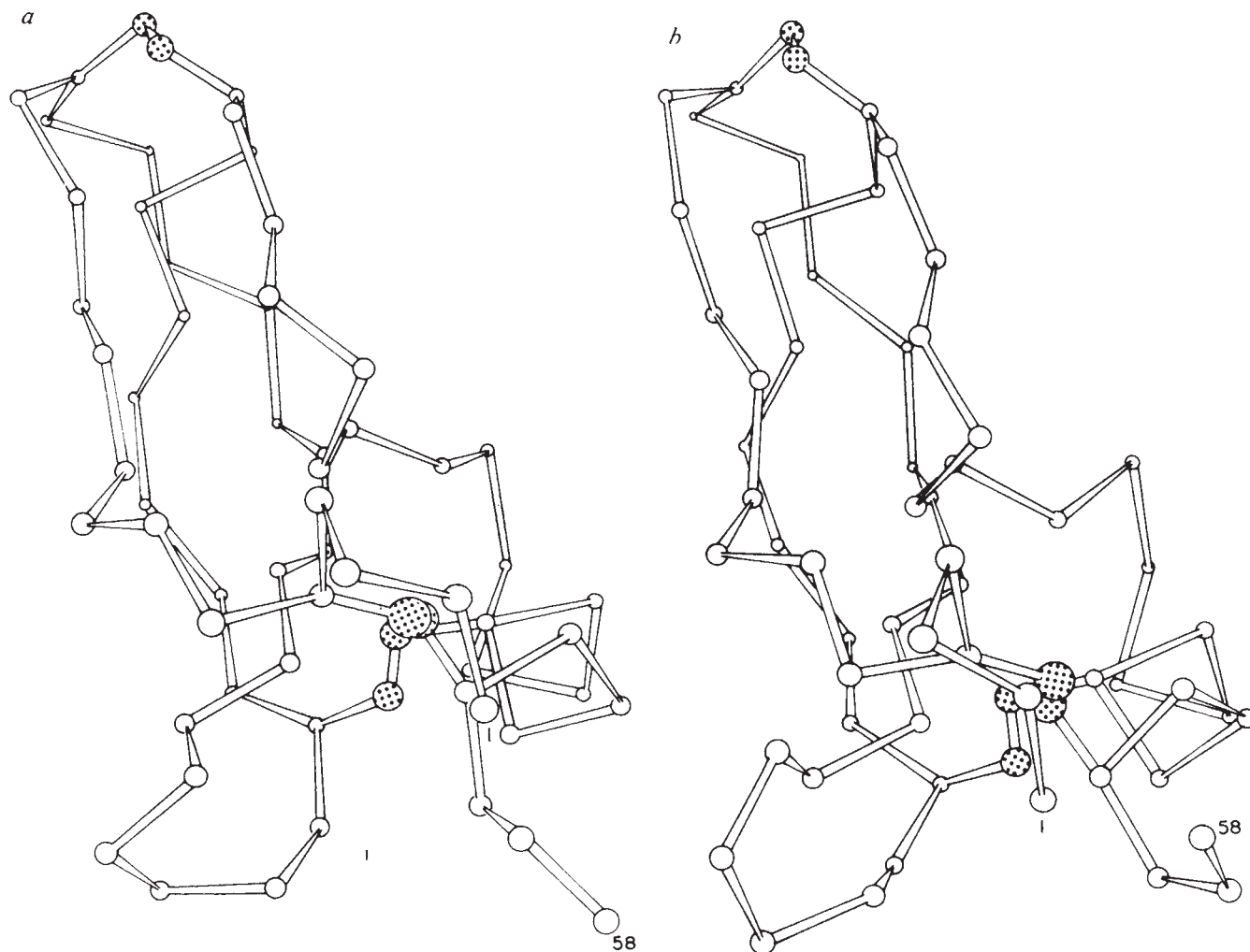


Fig. 1 The peptide backbone (α carbons) and disulphide bonds of PTI. *a*, X-ray structure²¹. *b*, Time evolved structure after 3.2 ps of dynamical simulation.

deviation of backbone atoms equal to 0.5 Å, with the largest changes at the surface of the protein. Also, a variety of other studies^{25–27} support the picture of a protein accommodating its structure to different environments. In any case, the X-ray and dynamic average structures are sufficiently similar that the present results should give a meaningful approximation to the short-time dynamics of PTI.

Figure 2 shows the r.m.s. displacements of the α carbons, relative to the dynamical average structure. The three residues at the carboxyl end of the chain have particularly large fluctuations; these are due to the presence of excess kinetic energy resulting from local strain in the initial structure. Some of this kinetic energy was transferred to the nearby amino end of the chain, which also exhibits relatively large r.m.s. displacements. The important fluctuations in residues 25–28 of the loop connecting the two strands of β sheet seem to reflect an intrinsic softness in this part of the molecule. The smaller fluctuations of the β sheet and α helical regions are also evident. The side-chain atom displacements are somewhat larger than those of the α carbons, but generally show a similar pattern of variation. The average r.m.s. fluctuation of all atoms is 0.9 Å. Particularly large r.m.s. displacements (1.8–3.1 Å) occur in the side-chains of Lys 26, Arg 39, Arg 42, Lys 46 and Met 52; all of these are at the surface of the protein. Overall, the large fluctuation regions correspond to those deviating most from the X-ray structure. In principle, the temperature factors from the X-ray analysis can be compared with the calculated fluctuations. The r.m.s. atomic fluctuation for the α carbons

obtained from the experimental temperature factor is 0.4 Å. Also, some of the more mobile regions correspond to groups of atoms which were not located or had unusually high temperature factors (J. Deisenhofer, personal communication).

The range of variation of the internal coordinates in the static structure can be compared with the dynamical averages and fluctuations. Since the X-ray results for bond lengths and angles correspond to standard values for the different amino acids²¹, we use for comparison the energy-refined geometry (ERG)¹⁸, which reflects the effect of the environment on the coordinates. Selected examples for backbone coordinates are listed in Table 1. It can be seen that the ERG and dynamical averages and r.m.s. variations are in close correspondence. It should also be noted that the average r.m.s. dynamical fluctuation of any given coordinate is two to five times larger than the variation in the coordinate throughout the molecule. The results for the side chains are similar. For the dihedral angle ω , neither the ERG refinement nor the dynamic results show any significant distortions or unusual fluctuations for residues 14–17; this differs from the X-ray results²¹.

The dynamic averages for individual ϕ and ψ dihedral angles differ from the corresponding ERG results by less than 15° for 70 of the 115 backbone angles and by somewhat larger values for the rest (up to 30°). The behaviour of the sidechain dihedral angles is similar to ϕ and ψ .

The r.m.s. dynamic fluctuations of the bond lengths, the bond angles, and the ω angles are essentially constant along the backbone except for slightly (~20%) larger fluctuations in

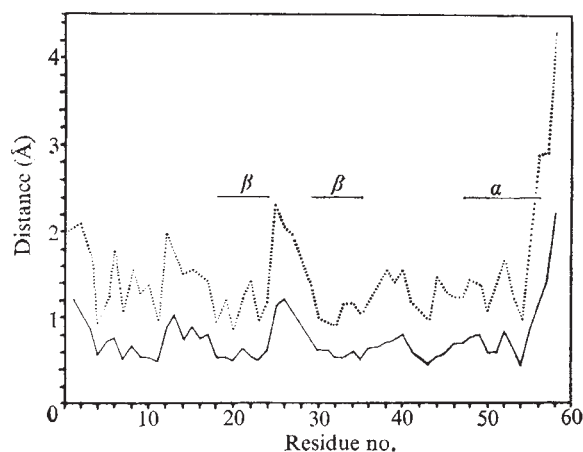


Fig. 2 R.m.s. displacements (—) and maximum displacements (....) of the α carbons, relative to the dynamical average structure.

residues 56–58. For the ϕ and ψ dihedral angles, the r.m.s. dynamic fluctuations range from 10° to 40° and show more variation along the chain; in both the β sheet and α helix regions these fluctuations are about 18° on the average, while in the loop region the fluctuations average about 25° .

Bond lengths calculated from the dynamically averaged set of Cartesian coordinates were uniformly 0.02 – 0.03 Å shorter than the average bond lengths obtained directly. A similar discrepancy arises in calculating interatomic distances from X-ray diffraction data because of thermal atomic motion²⁸.

Time-dependence of motions

The fact that the individual PTI internal coordinates exhibit dynamic fluctuations of appreciable magnitude raises questions concerning the details of the motions, including their time evolution and spatial correlation.

To illustrate the time-dependence of individual coordinate fluctuations, we present results obtained for the Phe 22. This residue is relatively well buried in the protein and so should exemplify the effect of interactions between different parts of the molecule; other residues examined were found to have qualitatively similar dynamic behaviour. Figure 3a shows the time-dependence of several Phe 22 internal coordinates; the considerable variation in the frequencies, magnitudes of fluctuations, and irregularities of the behaviour of the different coordinates is evident.

To quantitate the average decay characteristics of fluctuations, so as to best analyse the coupling between the coordinate and the rest of the molecule, it is helpful to introduce time

correlation functions²⁹. The time correlation function $C_A(t) = \langle A(\tau+t)A(\tau) \rangle$, for a dynamical variable A is obtained by multiplying the value of $A(\tau)$ by $A(\tau+t)$, the value taken by A after the system has evolved for an additional time t , calculating such products for a representative set of initial times τ and averaging. If A is the fluctuation of a variable from its mean value, $\langle [A(\tau)]^2 \rangle$ is the mean-square fluctuation of the variable for an equilibrated system, while the time correlation function $C_A(t)$ describes the average decay behaviour of the fluctuation. Time correlation functions for the fluctuations of the internal coordinates of Phe 22 shown in Fig. 3a are given in Fig. 3b. Fluctuations of the backbone bond length $N_{22}-C_{22}^{\alpha}$ exhibit a partial loss of correlation in the first 0.1 ps, followed by a very slow decay of the residual small amplitude oscillation. Other backbone and sidechain bond lengths behave similarly. Although the specific oscillation frequencies depend on the force constants and the masses of the bonded atoms, all have long-lived oscillations; among others, the disulphide bond $S_{14}-S_{38}$ was examined and showed no atypical features. The other stiff internal coordinates (bond angles, dihedral angle ω) also exhibit partial loss of correlation in the first 0.1 ps but have persistent though less regular small-amplitude oscillation at longer times.

The relaxation behaviour can be analysed further by obtaining the Fourier transform of the correlation function and finding the dominant frequency components. For the $N_{22}-C_{22}^{\alpha}$ bond, the main peaks are found at 800 cm^{-1} , $1,010$ cm^{-1} , $1,110$ cm^{-1} , $1,170$ cm^{-1} , and $1,430$ cm^{-1} . These frequencies correspond to the calculated normal modes that involve the largest displacements of the $N-C^{\alpha}$ bond length of the isolated fragment $\text{CH}_3-\text{CO}-\text{Phe}-\text{NH}-\text{CH}_3$ (787 ; 952 ; $1,099$; $1,161$; and $1,425$ cm^{-1}). In addition, there are relatively small contributions from many low frequency motions. Thus, the $N_{22}-C_{22}^{\alpha}$ bond fluctuations are those expected for a strongly coupled system of local oscillators that is weakly coupled to the rest of the protein molecule. For the bond angles and the dihedral angle ω , there are important frequency contributions in the range 300 – $1,200$ cm^{-1} , which correspond to the fragment vibration frequencies associated with these degrees of freedom. Since frequencies below 300 cm^{-1} are also important, it seems that the bond angle and the dihedral angle ω fluctuations are significantly coupled to the protein matrix. Backbone dihedral angles (ϕ , ψ) are seen in Fig. 3b to exhibit considerable damping, though ϕ_{22} preserves long term correlation in a low frequency oscillation of the order of 2 ps. For the sidechain dihedral angle, χ_{22}^2 , associated with the rotation of the phenyl ring, damping produces a nearly monotonic decay with a relaxation time of 0.2 ps. It is clear from the decay behaviour of the correlation functions that the fluctuations of the ϕ , ψ and χ dihedral angles are dominated by interactions with the protein matrix. It is reasonable that torsional motions which involve substantial displacements of large groups inside proteins

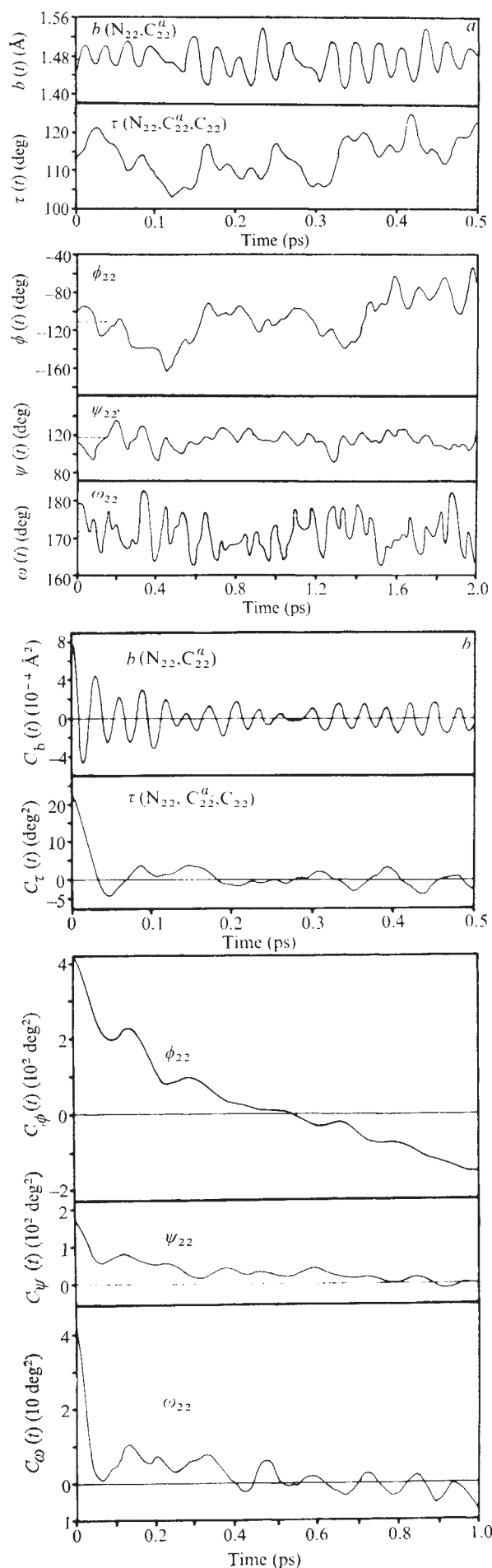
Table 1 Backbone internal coordinates*

Internal coordinate	Average†		r.m.s. variation†		Average dynamical r.m.s. fluctuation‡
	ERG	Dynamics	ERG	Dynamics	
Bond lengths					
N–C ^α	1.476	1.478	0.005	0.004	0.028
C ^α –C	1.531	1.533	0.004	0.004	0.027
C–O	1.237	1.237	0.002	0.003	0.015
C–N	1.321	1.323	0.003	0.003	0.018
Bond angles					
C–N–C ^α	124.6	125.8	1.6	1.7	4.8
N–C ^α –C	113.8	114.9	2.9	2.4	4.8
C ^α –C–N	115.4	114.6	1.8	1.7	4.6
C ^α –C–O	119.7	118.7	1.5	1.5	4.6
Dihedral–angle					
ω	177.9	177.8	5.8	4.7	8.5

* Units are Å for bond lengths, degrees for bond and dihedral angles.

† These values represent the average and r.m.s. variation over the 58 amino acids of a given type of coordinate.

‡ These values represent the r.m.s. dynamical fluctuation of the individual coordinate averaged over the 58 amino acids.



(for example, aromatic sidechains) will have a collective, diffusion-like behaviour, while those subject to smaller steric interactions will retain more of a local character.

The relaxation of individual atom fluctuations seems to be dominated by rotations of the dihedral angles. Atom displacement time correlation functions exhibit qualitative features and characteristic times similar to those shown for the dihedral angles. In many cases, the correlation functions show nearly monotonic decay with relaxation times of the order of a ps.

Correlated fluctuations

The large fluctuations found in the individual dihedral angles might seem to suggest a degree of structural mobility that is greater than that corresponding to the r.m.s. fluctuations of the individual atoms. In fact, the fluctuations of neighbouring dihedral angles are generally correlated so as to minimise disturbances of the backbone and sidechain atoms. For the typical backbone dihedral angle ϕ_{33} , which is at the centre of a strand of β sheet, the observed equal-time correlations of fluctuations of neighbouring ϕ angles and of neighbouring ψ angles are shown in Fig. 4. The large negative correlation $\langle \Delta\phi_{33}(\tau)\Delta\psi_{32}(\tau) \rangle$ is striking and has as a consequence the conservation of the general direction of the backbone and of the approximate position of the sidechains; the larger the individual dihedral fluctuations, the stronger the anti-correlation $\langle \Delta\phi_i(\tau)\Delta\psi_{i-1}(\tau) \rangle$ is found to be. The correlation pattern seen in Fig. 4 is similar to that obtained for an isolated harmonic α helix³⁰. A corresponding pattern of positive and negative dihedral angle correlations is likely to occur generally in strongly conserved regions of secondary structure. The anti-correlation $\langle \Delta\phi_i(\tau)\Delta\psi_{i-1}(\tau) \rangle$ is, in fact, present in other parts of the protein, though somewhat weaker outside the β sheet and α helical regions.

Structure-conserving correlations between dihedral angles also exist in the sidechains. For the eight aromatic sidechains with dihedral angles χ^1 and χ^2 , the normalised correlations $\langle \Delta\chi^1\Delta\chi^2 \rangle / \langle (\Delta\chi^1)^2 \rangle^{1/2} \langle (\Delta\chi^2)^2 \rangle^{1/2}$ range from -0.05 to -0.63; again, dihedral angles with larger r.m.s. fluctuations tend to be more strongly anti-correlated. This correlation is the one required to conserve approximately the orientation of the aromatic rings with respect to the polypeptide backbone.

Concerted motions

In addition to the structure-preserving correlations, a number of concerted atom motions were observed. One of these involves the relatively flexible loop region (residues 25-29) which seems to oscillate as a whole with a period of approximately 6 ps, as well as undergoing changes in shape (see Fig. 1a, b). Some damping of this motion would be expected in solution due to the high solvent accessibility of the loop.

Time correlation functions involving different internal coordinates (for example, $\langle A(\tau+t)B(\tau) \rangle$) can be used to probe the dynamical character of non-local, collective motions in the protein. One such case concerns time correlations between the fluctuations of the dihedral angle ϕ_{33} in the β sheet and the fluctuations of other backbone and sidechain dihedral angles in the molecule. The results show that both strands of the β sheet and some additional residues participate in a low-frequency 'rippling motion' ($\nu \approx 15 \text{ cm}^{-1}$); within each strand of the β sheet, backbone dihedral angles tend to oscillate with the phase relationships suggested by Fig. 4. Examination of the correlation functions for the atoms involved also shows damped oscillations of the same frequency.

A feature of the dynamics is that certain groups in the molecule undergo transitions from one minimum to another. Such behaviour was found for a number of sidechains;

Fig. 3 a, Time development of selected Phe 22 internal coordinates; the initial time is 4.9 ps after the beginning of the dynamical simulation; average values are indicated by broken line segments. b, Time correlation functions for fluctuations of selected Phe 22 internal coordinates.

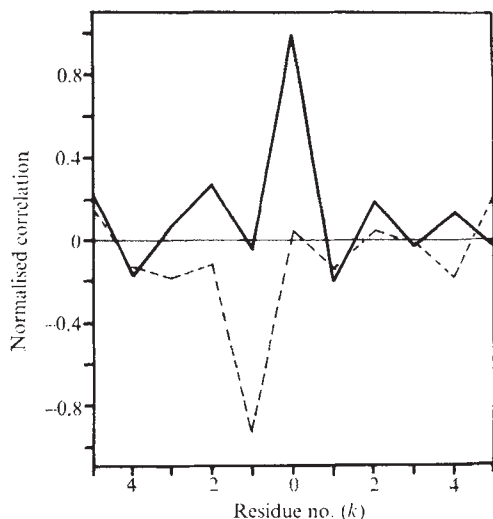
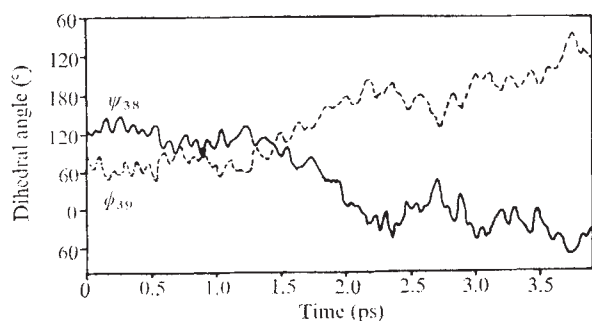


Fig. 4 Normalised equal-time correlations of fluctuations in ϕ_{33} with fluctuations in ϕ_{33+k} (—) and with fluctuations in ψ_{33+k} (---); the quantities plotted are, for example, $\langle \Delta\phi_{33}\Delta\phi_{33+k} \rangle / \langle (\Delta\phi_{33})^2 \rangle^{1/2} \langle (\Delta\phi_{33+k})^2 \rangle^{1/2}$.

for example, Met 52, where χ_{52}^2 moved successively from $-60^\circ \rightarrow +60^\circ \rightarrow -60^\circ \rightarrow 180^\circ \rightarrow 60^\circ \rightarrow 180^\circ$ and Arg 39, where χ_{39}^2 moved successively from $-60^\circ \rightarrow 180^\circ \rightarrow 60^\circ$. The times involved in these rotations vary between 0.2 and 2 ps. Since these sidechains are on the surface of the molecule, they have low barriers due to the protein; their detailed dynamics would be expected to be altered by interactions with the solvent.

In addition to the sidechain reorientations, there occurred a few concerted transitions in the backbone involving ψ_{i-1} , ϕ_i dihedral angle pairs. In these transitions, the anti-correlated angles ψ_{i-1} and ϕ_i rotate in the opposite sense by about 180° so that the intervening amide group flips over, but the backbone and sidechain directions are only slightly altered. The pairs involved are ψ_{38} , ϕ_{39} (near the 14–38 disulphide bond); ψ_{44} , ϕ_{45} ; and ψ_{56} , ϕ_{57} . All of these amide groups are located near the surface of the protein, so that the probability and dynamics of their transitions could be affected by solvent. Nevertheless, it is worthwhile to examine the behaviour since it can serve as a model of structural alterations in the presence of the protein matrix. Figure 5 shows the results for the ψ_{38} , ϕ_{39} pair. The transition is seen to take place in about 1 ps. During this period, the variation in the angles is roughly linear in time and the kinetic energy of the amide group does not show any significant departure from the thermal value. The amide flip is seen to be followed by a conformational adjustment period of 3–4 ps, during which ψ_{38} , ϕ_{39} drift over a relatively small angular interval into their new equilibrium ranges. It is apparent that there is a smooth change from one minimum to the other, while the protein relaxes so that the transition can occur without a large activation energy. The details of the driving torques and other matrix effects remain to be analysed, as do a variety of other larger scale motions that occur.

Fig. 5 Amide group transition; the time development of ψ_{38} and ϕ_{39} during the transition is shown.



Discussion

A molecular dynamics study of the pancreatic trypsin inhibitor has revealed a rich variety of motional phenomena that occur on the atomic level at ordinary temperatures. PTI has been shown to exhibit significant structural fluctuations, and detailed information on the magnitude, correlations and decay of these fluctuations has been obtained.

One important result of the dynamical simulation is that many aspects of the internal motion of PTI suggest that the folded protein is fluid-like at ordinary temperatures³¹. By this we mean that the fluctuations in atom positions, although confined to the neighbourhood of the average structure, have a diffusional character. In other words, the dynamics of atomic displacements are dominated by collisions with neighbouring atoms, at least on the picosecond time scale.

Also of significance is the heat capacity of the protein, which may be calculated from the magnitude of fluctuations in the kinetic energy³². Over the final 2 ps of the simulation, these fluctuations had the average value $\langle (\Delta KE)^2 \rangle = 87 \text{ kcal}^2 \text{ mol}^{-2}$; the corresponding heat capacity per atom is $C = 2.3 k$, where k is Boltzmann's constant. This result, which is midway between those expected for a harmonic solid ($C = 3k$) and for a hard-sphere fluid ($C = 1.5k$) indicates that the potential governing the atomic motions is significantly anharmonic. Similar values have been measured in real fluids; for example, at 84 K in liquid argon, $C = 2.32k$ (ref. 33). The calculated heat capacity of $0.33 \text{ cal g}^{-1} \text{ K}^{-1}$ is consistent with experimental values for proteins¹³.

An idealised model for proteins is that of a dense hard-sphere fluid composed of particles that are connected by flexible links. This implies that many of the dynamical properties (though not necessarily the correct average structure) can be obtained from any potential function which includes the forces that depend strongly on distance (covalent and hydrogen bonds, non-bonded repulsions) and provides sufficient attractive interactions to preserve the compact structure of the native system. Such a representation of a protein has much in common with the hard-sphere model of dense fluids, which has had considerable success recently in reproducing their equilibrium and dynamic properties³⁴. The close correspondence of the protein dynamics with that of the high temperature (300 K) solid phase of normal alkanes is also of interest^{35,36}. The importance of motions in which hard-sphere interactions dominate suggests that in conformational changes the non-bonded contacts will determine the transmission of effects from one part of the molecule to another. One such case of biological interest is the recent determination of the mechanism of tertiary structural change in a haemoglobin subunit, where non-bonded contacts were found to have the dominant role³⁷.

Although the time scale of the simulation is rather short, it provides essential details concerning the dynamics that should be of considerable utility in analysing longer time scale phenomena. A basic result is the r.m.s. atom fluctuation of $0.9 \text{ \AA}$. In a stochastic model that assumes independent atom motions, this provides a means for estimating the probability of larger fluctuations. Of corresponding significance is the nearly monotonic decay of the atom fluctuations with a time constant of $\sim 1 \text{ ps}$. It is also of interest that the kinetic energy of residues 49–58 remains 10–20% above the equipartition value for more than 9 ps; this indicates that vibrational energy can localise for relatively long time periods in proteins; this may be compared with the vibrational relaxation times in polyatomic fluids, which are often of the order of many picoseconds, even when efficient relaxation pathways exist³⁸.

It is clear from the dynamic simulation that the protein with room temperature kinetic energy samples highly anharmonic regions of the potential surface. Further, from an examination of the details of the coordinates during the run, as well as the dynamic average structure, it follows that the protein is able to wander over a range of conformations that are in the neighbourhood of the X-ray structure but not identical with it. The protein

seems to be oscillating in a confined multidimensional space, as shown by the fact that the fluctuations from the average structure are similar during the entire dynamical run. Some of the fluctuations that involve reorientation of sidechains or localised portions of the backbone may represent local minima, though their detailed characteristics have not yet been investigated.

Two limitations of the present model need to be mentioned. First is the approximate nature of the potential energy function. Although in previous applications this function has proved reliable for energy refinement of the PTI structure and the calculation of vibrational spectra of small peptides, some of the details of dynamical simulations of proteins may be sensitive to the choice of potentials. Also, hydrogen atoms have not been explicitly included in the simulation. Their characteristically high frequency motions suggest that replacing them by what can be regarded as an effectively averaged potential may not be a severe approximation; nevertheless, there remains a question as to whether their detailed consideration could introduce somewhat more rigidity into the close-packed structure.

Another limitation of the model is the neglect of solvent. The structure and dynamics of groups at the protein surface are expected to be influenced by hydrogen-bond formation and collisions with solvent molecules, as well as by hydrophobic effects that vary with the exposed area. But, many of the essential features of the internal motions analysed in the dynamic simulation of PTI are not expected to be grossly altered by the presence of solvent. The electrostatic component of the potential energy was found to vary over a smaller range than some other energy components during most of the run, and there was no marked low-frequency alteration of the overall shape of the protein during the simulation. Large variations in these properties, which might be found on a longer time scale, would require consideration of dielectric and hydrodynamic effects, respectively.

It is most important to have available detailed tests of the dynamic simulation. Most closely related are results obtained from fluorescence depolarisation, NMR, fluorescence quenching and hydrogen exchange studies. Measurements of fluorescence depolarisation on a picosecond time scale could be interpreted directly by use of the dynamic results. Other data (for example, fluorescence quenching) require assumptions, such as that of stochastic behaviour, to use the information on fluctuations that has been obtained. For certain rare phenomena that are not represented in the dynamic simulation because they involve high activation barriers (for example, rotation of a tyrosine

sidechain), special methods based on dynamics starting at the transition state may have to be introduced^{39,40}. Comparisons with a variety of experimental data, as well as studies of motional effects on enzyme mechanisms and of conformational changes involved in ligand binding are in progress. A combination of these theoretical approaches with the interpretation of related experiments will provide a unified description of motions in proteins.

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- ¹ Brown, K. G., Erfurth, S. C., Small, E. W. & Peticolas, W. L. *Proc. natn. Acad. Sci. U.S.A.* **69**, 1467-1469 (1972).
- ² Hull, W. E. & Sykes, B. D. *J. molec. Biol.* **98**, 121-153 (1975).
- ³ Campbell, I. D., Dobson, C. M., Moore, G. R., Perkins, S. J. & Williams, R. J. P. *FEBS Lett.* **70**, 96-100 (1976).
- ⁴ Lakowicz, J. R. & Weber, G. *Biochemistry* **12**, 4171-4179 (1973).
- ⁵ Savitt, M. L. & Galley, W. C. *Proc. natn. Acad. Sci. U.S.A.* **71**, 4154-4158 (1974).
- ⁶ Eftink, M. R. & Ghiron, C. A. *Biochemistry* **15**, 672-680 (1976).
- ⁷ Ghose, R. C. & Englander, S. W. *J. biol. Chem.* **249**, 7950-7955 (1974).
- ⁸ Vas, M. & Boross, L. *Eur. J. Biochem.* **43**, 237-244 (1974).
- ⁹ Yguerabide, J., Epstein, H. F. & Stryer, L. *J. molec. Biol.* **51**, 573-590 (1970).
- ¹⁰ Holowka, D. A. & Cathou, R. E. *Biochemistry* **15**, 3379-3390 (1976).
- ¹¹ Suezaki, Y. & Go, N. *Int. J. Peptide Protein Res.* **7**, 333-334 (1975).
- ¹² McCammon, J. A., Gelin, B. R., Karplus, M. & Wolynes, P. G. *Nature* **262**, 325-326 (1976).
- ¹³ Cooper, A. *Proc. natn. Acad. Sci. U.S.A.* **73**, 2740-2741 (1976).
- ¹⁴ Lipscomb, W. N. *Acc. Chem. Res.* **3**, 81-89 (1970).
- ¹⁵ Perutz, M. *Nature* **228**, 726-739 (1970).
- ¹⁶ Huber, R., Deisenhofer, J., Colman, P. M., Matsushima, M. & Palm, W. *Nature* **264**, 415-420 (1976).
- ¹⁷ Karplus, M. & Weaver, D. L. *Nature* **260**, 404-406 (1976).
- ¹⁸ Gelin, B. R. & Karplus, M. *Proc. natn. Acad. Sci. U.S.A.* **72**, 2002-2006 (1975).
- ¹⁹ Rahman, A. *Phys. Rev. A* **2**, 405-411 (1964).
- ²⁰ Huber, R., Kukla, D., Rühlmann, A., Epp, O. & Formanek, H. *Naturwissenschaften* **57**, 389-392 (1970).
- ²¹ Deisenhofer, J. & Steigemann, W. *Acta Crystallogr.* **B31**, 238-250 (1975).
- ²² Gear, C. W. *Argonne Nat'l. Lab. Rep. No. ANL-7126* (1966).
- ²³ Imoto, T., Johnson, L. N., North, A. C. T., Phillips, D. C. & Rupley, J. A. in *The Enzymes III* (ed. Boyer, P. D.) (Academic, New York, 1972).
- ²⁴ Moul, J. et al. *J. molec. Biol.* **100**, 179-195 (1976).
- ²⁵ Yu, N.-T. & Jo, B. H. *J. Am. Chem. Soc.* **95**, 5033-5037 (1973).
- ²⁶ Austin, R. H., Beeson, K. W., Eisenstein, L., Frauenfelder, H. & Gunsalus, I. C. *Biochemistry* **14**, 5355-5373 (1975).
- ²⁷ Stuhmann, H. B. *J. molec. Biol.* **77**, 363-369 (1973).
- ²⁸ Busing, W. R. & Levy, H. A. *Acta Crystallogr.* **17**, 142-146 (1964).
- ²⁹ Zwanig, R. A. *Rev. Phys. Chem.* **16**, 67-102 (1965).
- ³⁰ Go, M. & Go, N. *Biopolymers* **15**, 1119-1127 (1976).
- ³¹ Fehder, P. L., Emeis, C. A., Futrelle, R. P. *J. chem. Phys.* **54**, 4021-4934 (1971).
- ³² Lebowitz, J. L., Percus, J. K. & Verlet, L. *Phys. Rev.* **153**, 250-254 (1967).
- ³³ Egelstaff, P. *An Introduction to the Liquid State* (Academic, New York, 1967).
- ³⁴ Chandler, D. *Acc. Chem. Res.* **7**, 246-251 (1974).
- ³⁵ Barnes, J. D. *J. chem. Phys.* **58**, 5193-5201 (1973).
- ³⁶ Peterline-Neumaier, T. & Springer, T. *J. Polym. Sci. (Phys.)* **14**, 1351-1359 (1976).
- ³⁷ Gelin, B. & Karplus, M. *Proc. natn. Acad. Sci. U.S.A.* **81**, 801-805 (1977).
- ³⁸ Laubereau, A. & Kaiser, W. A. *Rev. Phys. Chem.* **26**, 83-99 (1975).
- ³⁹ Anderson, J. B. *J. chem. Phys.* **58**, 4684-4692 (1973).
- ⁴⁰ Bennett, C. H. in *Diffusion in Solids* (eds Burton, J. J. & Nowick, A. S.), 73-113 (Academic, San Francisco, 1975).

The rings of Uranus: theory

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The structure of the recently discovered rings of Uranus is accounted for by a series of orbital resonances with the satellites Ariel, Titania and Oberon. The observations and their interpretation will considerably improve our knowledge of the mass of the planet and its dynamical oblateness and could influence our ideas on the formation of planets and satellites.

THE recently discovered rings encircling the planet Uranus¹ are the second example we have of a planetary ring system; however, the rings of Saturn and those of Uranus have quite different structures, and different theoretical discussions will be required. In each case resonant interactions, between the particles making up the rings and the perturb-

ing satellites, have to be considered. But in the case of Saturn the rings are wide, and a continuum of orbital periods of the circulating particles exists; the sharply defined orbits for which resonance effects with the satellites can be invoked concern the divisions between the rings. One has to suppose then that a profuse supply of particulate matter was available to populate a disk surrounding the planet, and that orbit-orbit resonances involving particles and satellites have caused certain lanes to be swept clear. In the case of the Uranus ring system the situation is in a sense the opposite: the rings themselves are exceedingly narrow and resonances can therefore not be responsible for defining them by any sweeping action. A set of rings 10 km wide with a radius of the order of 45,000 km must be defined by conditions that lend a stability peculiar to the ring

orbits alone. Resonances with satellites must provide here the explanation for the presence or great concentration of the orbiting material in exceedingly narrow lanes.

An explanation in terms of celestial mechanics of any observed orbit-orbit resonance must have two components: one is the stability of the motion maintaining the precise relationship of periods that is seen; the other is a mechanism for setting up the particular resonance. If only gravitational forces are invoked the system is strictly conservative and the precise condition of a resonance cannot be expected to be reached. Liouville's theorem applies and material cannot become concentrated in the particular domain of phase space represented by a resonance. For this reason other orbital resonances in the Solar System have had explanations involving the operation of slight but significant non-conservative forces, namely the tidal drag between a satellite and its parent planet. It is the secular changes resulting from the drag-forces that drive the system through the various numerical relationships, with the possibility then of being arrested in one of them. The requirement is for the presence of a drag-force small enough so that the slight effects of a resonance can arrest the secular evolution, yet large enough to drive it through some resonance conditions in the time available. It is the lengths of the time-spans that have been available in the solar system that have made it possible for so many systems to be channelled into resonances.

In the case of particles, tidal forces are negligibly small, and another drag force is required. The case that the Poynting-Robertson effect (light-drag) on small particles will feed them into resonant orbits has been discussed before² and seems applicable to the case of Uranus' rings. We thus envisage a supply of small particles having been available in the neighbourhood of Uranus, and the action of the Poynting-Robertson drag (chiefly due to sunlight) having been responsible for driving them gradually, on spiralling orbits, towards the planet. It is understood that such a swarm would settle down, through internal friction, to a common plane, and that this plane would adjust itself to coincide with the equatorial plane of an oblate planet. In the course of this slow spiralling, resonances with the satellites of Uranus will be encountered. At such resonances, when certain precise numerical relations hold between the mean periods of the satellites and of the particles in the disk, it is possible for angular momentum to be transferred between them. A stable condition can then occur when the angular momentum that a particle loses by the Poynting-Robertson effect is just compensated by the angular momentum supplied by the satellites in the resonance.

The search for the appropriate resonances accounting for the rings of Uranus was guided by the following considerations. First, the orbits of the particles slowly spiralling towards Uranus will be closely circular and will lie in the equatorial plane of the planet. The satellites of

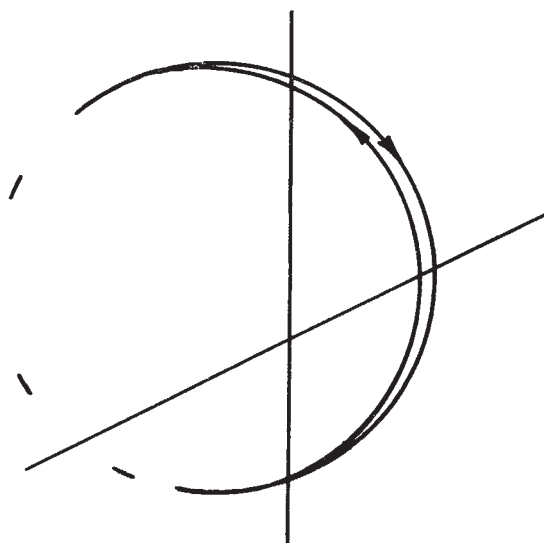


Fig. 1 Libration of the ring particles about a stable longitude is expected, and this would produce a longitudinal dependence of the ring's opacity. The arrows mark the paths of the librating particles in a coordinate frame centred on the planet but rotating with the resonant mean motion. The straight lines represent arbitrary apparent paths of the occulting star projected onto the orbital plane of the particles. The fact that each ring is caught in a different phase at the occultation will account for some being seen on one side of the planet only and some on both.

Uranus also have orbits that are nearly circular and in the equatorial plane (with the exception of the tiny Miranda³). In such circumstances resonance between two bodies has no dynamical significance. This is because in a circular, coplanar system of orbits there are no reference points (apses or nodes), and thus all orbital configurations are equally stable and the system is degenerate. Resonances involving three bodies—a ring particle and two satellites—do not have this degeneracy as one of the satellites can act as a reference point for the other two. In effect, the satellite takes over the role that an apse or a node has in two-body resonance. The precise condition for three-body resonance is

$$qn_1 - (q+p)n_2 + pn_3 = 0 \quad (1)$$

where the n_1 are mean motions (suffix 1 refers to the ring particle) and p and q are integers. It follows from equation (1) that in a reference frame rotating with the mean motion of the ring particle, the mean motions of the satellites are commensurate in the ratio $p : (p+q)$.

The strength of such resonances, that is, for our purposes, the amount of angular momentum that can be transferred to the ring particle, is known to depend chiefly on: the order of the resonance, q , (which must be low and preferably unity); the product of the satellite masses; and on a certain function of their orbital radii^{4,5}. From estimates of the function masses based on their visual magnitudes⁶ one would suppose that the combinations of Ariel and Titania and of Ariel and Oberon would dominate (see Table 1). Discounting the tiny Miranda, Ariel is the satellite closest to Uranus.

In comparing the observational data on the rings with predictions based on resonance theory, we consider that the outer two obscuring events, one seen on one side of the planet and one at a different distance on the other, are two separate rings. As the particles in a resonant ring are expected to be librating about a stable longitude (displaced from the longitude of conjunction of the perturbing satellites by an angle dependent on the mean torque that needs to be supplied by the resonance to offset the light drag), the particle distribution in the ring will

Table 1 Products of satellite masses

Satellites	$m_2 m_3$ (Planetary units $\times 10^{-11}$)
Miranda, Ariel	19
Miranda, Umbriel	5
Miranda, Titania	32
Miranda, Oberon	25
Ariel, Umbriel	99
Ariel, Titania	580
Ariel, Oberon	447
Umbriel, Titania	170
Umbriel, Oberon	131
Titania, Oberon	770

have a strong longitudinal dependence (see Fig. 1). In such circumstances, the characteristics of the obscuring events will depend on the phase in which each ring is caught by the occultation. It is only at the time of conjunction of the pair of satellites responsible for a set of rings that these would have a common orientation. Thus, one particular path could produce two narrow events of similar opacity, but a path passing near the centre of libration could produce a broader and more intense event on one side of the planet (possibly even with a double peak, as observed for the outer two rings) and nothing at all on the other. We thus have to account for six rings, whose radii have the distinctive pattern of three pairs.

The two sets of preliminary estimates of the radii of the six rings, as calculated by Elliot *et al.*¹ and Marsden⁷, and the radii of the resonant orbits for the dynamically more significant pairs of satellites, as calculated from equation (1) with $q=1$, are shown in Fig. 2. It is immediately apparent from this figure that the distinctive pattern of the rings is reproduced well by the entire class of first-order resonances that fall in that interval and are associated with Ariel and Oberon and Ariel and Titania—the two pairs of satellites which, chiefly on the basis of the mass products shown in Table 1, we expect to have the greatest dynamical significance.

The systematic differences of several hundreds of kilometres between the two estimates of the ring radii are due to the fact that neither authors^{1,7} have yet made complete allowance for the motion of the Kuiper Airborne Observatory (KAO) and because the offset of the path of the occulting star from the centre of Uranus is imperfectly known. As the separation of the rings is small compared with their radii, however, the errors will be proportional to the radii, and we should expect a plot of one set of estimates against the other to be linear. By the same argument, a plot of each set of observations against the radii predicted by the resonances should also be linear, if indeed the proposed identifications of the resonances are correct. The observed linearity of these plots (see Fig. 3) therefore provides a strong argument in favour of the identifications.

The root mean square deviations from the least squares fits shown in Fig. 3 are 66 km in each case. As Marsden rounded off his estimates of the ring radii to the nearest hundred kilometres we can conclude that the fit of the points to a straight line is as close as could be expected, and thus different from our predictions only by a systematic offset. Elliot *et al.*¹ gave distances of the rings from the centre

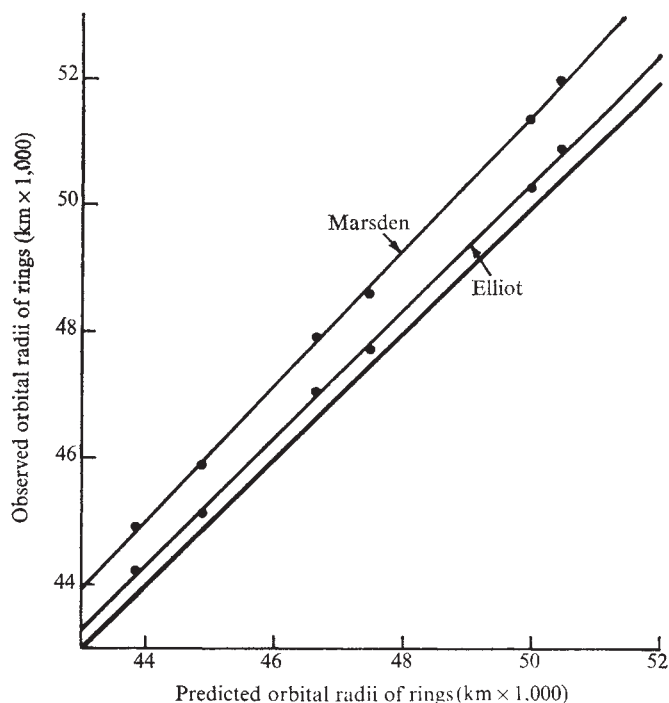


Fig. 3 Preliminary estimates of the radii of the observed rings, as calculated by Elliot *et al.*¹ and Marsden⁷, are plotted against the radii of the resonant orbits of Ariel and Oberon and Ariel and Titania. These radii, and the deviations from the above least squares straight line fits, are also listed in Table 2. A line of slope unity is shown for comparison. The quality of the straight line fit in each case indicates that a very consistent offset rather than a random relation exists between both sets of observational data and the radii predicted by the resonances.

of Uranus before immersion as well as after, and thus from their paper we can obtain two estimates of the ring separations which are independent of the assumed occultation midtime. These are listed in Table 3. The root mean square difference in the separations is 49 km. These differences would seem to be too large to be accounted for by any errors in the data reduction and would therefore indicate that the rings probably have eccentricities and inclinations of the order of 0.001 (these slight departures from exact circularity and coplanarity being generated by the resonant interactions between the ring particles and the perturbing satellites). But, again, we conclude that the straight line fit is as good as could be expected with the present level of observational uncertainty. We therefore conclude that the structure of the rings of Uranus has been determined by three-body resonances with the satellites Ariel, Titania and Oberon.

It seems not to have been noted before that these same three satellites are remarkably close to a first-order resonance condition themselves. In a frame rotating with Ariel, the innermost of the three, the ratio of the relative mean

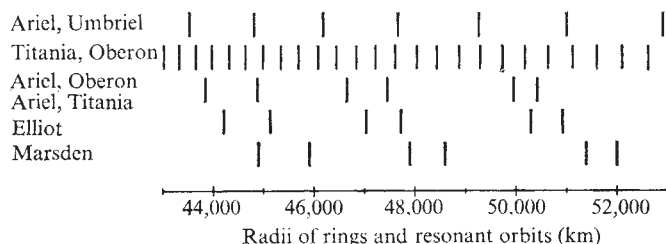


Fig. 2 The two sets of preliminary estimates of the ring radii (labelled Elliot and Marsden) were calculated using the same set of occultation times. The systematic differences of several hundreds of kilometres are due to the fact that neither Elliot *et al.*¹ nor Marsden⁷ have yet made complete allowance for the motion of the Kuiper Airborne Observatory, and because the offset of the path of the occulting star from the centre of Uranus is imperfectly known. Resonant orbits for the dynamically more significant pairs of satellites are shown above the ring radii. The resonant orbits of Ariel and Oberon and of Ariel and Titania are shown on the same line for comparison of their distinctive pattern of three pairs with the observations; from left to right the first in each pair is the Ariel–Oberon and the second the Ariel–Titania resonance; the exact radii are listed in Table 2.

Table 2 Radii of resonant orbits

Satellites	p	q	Radius* (km)	Deviations (km)	
				Elliot ¹	Marsden ⁷
Ariel, Titania	9	1	50,433	+78	+65
	10	1	47,451	–88	–119
	11	1	44,875	–58	–41
Ariel, Oberon	8	1	49,950	–48	–14
	9	1	46,651	+54	+44
	10	1	43,849	+60	+65

*Assuming $J_2 = 0.012$

Table 3 Distances of rings from centre of Uranus*

Pre-immersion occultations	Ring separation, S	Post-emersion occultations	Ring separation, S'	$S-S'$
44,144		44,258		
	962		890	+72
45,106		45,148		
	1900		1939	-39
47,006		47,087		
	678		665	+13
47,684		47,752		

*The distances shown correspond with the values shown in Elliot, Dunham and Mink's Figure and were calculated from the times of the obscuring events quoted by Elliot *et al.*¹, assuming: a constant shadow velocity of 11.7 km s^{-1} ; a constant location on Earth; 25,900 km for the radius of Uranus; and an occultation midtime of 21 h 06 min UT.

motions of Titania and Oberon is 0.87414, differing from $7/8$ by one part in 10^3 . This close proximity to a similar resonance ($q = 1$, $p = 7$) as those of the rings suggests that Ariel may have formed from a similar ring, while the rings now remaining are inside or close to Roche's limit. Perhaps other satellites can be found in the locations given by other values of p in the same series.

Positional observations at the distance of Uranus performed with optical telescopes were previously limited to approximately 0.1 arc s, or 1,300 km. The cutting points of the rings and of the disk of Uranus in the occultation can, however, be determined to a much greater accuracy, possibly as good as 3 km (or 2.3×10^{-4} arc s). The identification of the resonances of the rings now allows their periods to be determined with the same precision as those of the satellites. Thus period and radius of orbiting objects will both be known to the new accuracy level, not limited as previously by the telescope resolution. Therefore it will be possible to improve the determination of the mass of Uranus by approximately two orders of magnitude. The knowledge of the planet's radius and its mean density would in any case have been greatly improved by the

occultation observation, but the knowledge of the resonance conditions will further improve these quantities and allow a better value to be obtained also for the dynamical oblateness, J_2 . With many more occultations that will now be useful, Uranus's system of satellites and rings may become the most accurately determined complex dynamical system.

The size distribution of the particles making up a ring of this type is determined by the magnitude of the drag forces and the strength of the resonance. If light-drag is responsible, then the strength of the resonance, which can be calculated by the methods of Greenberg⁷ and Sinclair⁴, will place a lower bound on the sizes of the trapped particles.

The fact that the Uranus system now provides at least six more examples of the type of three-body resonance described here, raises again the question of the importance that this orbital configuration may have had in the course of the evolution of the Solar System. Dermott (ref. 8 and in preparation) has shown that in the Solar System as a whole, that is, in the planetary system and in the satellite systems of Jupiter, Saturn and Uranus, there is a strong preference for pairs of mean motions, expressed in the rotating system of a third body, to be close to a first-order commensurability. We believe that we can account for the origin of the rings of Uranus by a mechanism of drag forces channelling material into the resonant orbits, as previously discussed by Gold². This suggests that this type of mechanism may have had an important role in the formation processes of the Solar System.

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¹ Elliot, J. L., Dunham, E. & Mink, D. *Nature* **267**, 328-330 (1977).

² Gold, T. *Icarus* **25**, 489-491 (1975).

³ Whitaker, E. A. & Greenberg, R. J. *Mon. Not. R. astr. Soc.* **165** (1973).

⁴ Sinclair, A. T. *Mon. Not. R. astr. Soc.* **171**, 59-72 (1975).

⁵ Greenberg, R. J. *Mon. Not. R. astr. Soc.* **173**, 121-129 (1975).

⁶ Dunham, D. W. thesis Yale Univ. (1971).

⁷ Marsden, B. G., *J.A.U. Circ.* 3051 (1977).

⁸ Dermott, S. F. *Nature phys. Sci.* **244**, 18-21 (1973).

A consistent description of the nuclear α -decay process

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The essential features of a new theory of α decay and the need for such a theory are reviewed. An approximate relationship between half life and α -particle energy is deduced and checked for the decay of even polonium isotopes. Detailed calculations of one-body widths and spectroscopic factors for the polonium decays are presented and the implications of the results are discussed. It is also shown that the result for the α decay of ^{212}Po is consistent with the reverse α -transfer reaction.

THE process in which a nucleus in its ground or an excited state breaks up spontaneously into an α particle and a residual nucleus is the oldest observed reaction in nuclear physics. Gamow¹ gave a qualitative explanation of this process in terms of the quantum mechanical tunnelling of the α particle through the potential barrier formed by the sum of the real attractive

nuclear interaction between the α particle and the residual nucleus and the Coulomb repulsion between them. Combined with early studies of α -particle scattering from nuclei, this led to the first estimates of the size of the nucleus². Further development of these ideas based on the R-matrix theory of nuclear reactions have made possible satisfactory predictions of relative decay rates³. There has, however, been considerable difficulty and uncertainty in obtaining reliable predictions of absolute decay rates for heavy nuclei. The resolution of this problem has now become important because of the controversy over the possible existence of superheavy nuclei⁴ since the chance of observing these nuclei depends rather critically on their half life for α decay^{5,6}.

Predicting absolute decay rates

The difficulties associated with the prediction of absolute decay rates have two aspects. The nuclear structure part of the problem is concerned with the extent to which the α particle is

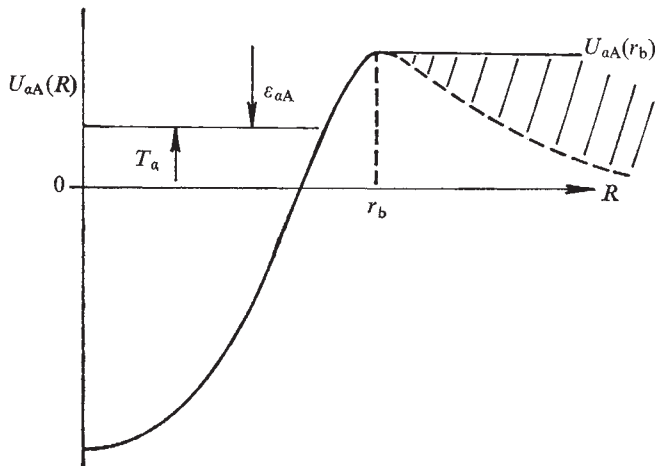


Fig. 1 The shape of the nuclear potential for the α particle in ^{212}Po .

performed in the nucleus; this is measured by the spectroscopic factor S_{α} which can be expressed as the ratio of the experimentally observed width Γ to the one-body width, that is

$$\Gamma = S_{\alpha}\Gamma_{\text{ob}}, \quad \Gamma = \hbar/\tau \quad (1)$$

where τ is the observed mean lifetime. Within the framework of the shell model it is particularly difficult to show convincingly how the initial shell model state evolves into the final state consisting of the residual nucleus and a free α particle, and in almost all theoretical work using this model the initial and final states are not orthogonal. The nuclear reaction part of the problem is concerned with the emission of the α particle. This is frequently treated by means of the R-matrix theory in which the width Γ of a narrow resonance is proportional to the barrier penetrability P , which in turn depends on the channel radius R_c . The theory does not determine the value of this radius beyond the rather general requirement that it should be sufficiently large to exclude coupling between the various exit channels, and it is well known that a relatively small change in this radius can change the penetrability by an order of magnitude⁷. An approximate method of calculating the penetrability is given by the WKB approximation but this method underestimates the penetrability by at least a factor of five^{8,9}. A further substantial uncertainty in the calculation of the penetrability arises from ambiguities in the phenomenological optical potential which describes the interaction of a free α particle with the residual nucleus^{7,8}.

We have developed a theory of α decay which eliminates all arbitrary radii and ambiguous potentials and provides a consistent description of the initial and final states. It yields spectroscopic factors in agreement with other data but in marked disagreement with the predictions of the shell model. Other authors have attempted such an approach; in one case¹⁰ the arbitrary radius was eliminated but not the potential ambiguities which introduced variations as large as 10^3 in the calculated widths, while more recent work¹¹ removed much of the uncertainty in the potential barrier but retained an arbitrary radius and introduced new uncertainties arising from limited knowledge of the absorptive part of the potential.

The first step is to represent the initial state Ψ_i as an eigenstate of an unperturbed Hamiltonian K so that the difference between K and the exact Hamiltonian H is the perturbation which leads to α decay. An expression for the width Γ of the decaying state can then be obtained using time-dependent perturbation theory or using the unified reaction theory of Feshbach. Both methods yield, to first order⁹

$$\Gamma_{\text{ob}} = 2\pi \int d\rho(E_f) |\langle \Psi_f | H - K | \Psi_i \rangle|^2 \quad (2)$$

where Ψ_f represents the final state with energy E_f and $d\rho(E_f)$ is

the density of states per unit energy interval. The initial and final states are described by the cluster model, instead of the shell model. In the final state the free α particle interacts with the nucleus through an optical potential and this is calculated using forms for the basic α -nucleon interaction and the nuclear matter distribution of the residual nucleus which are in agreement with other experimental data. The height of the real potential barrier and the position r_b of the peak of the barrier are well determined from low energy α -particle scattering and consequently provided an important test of the calculated potential. For the initial system a bound state is artificially created by continuing the potential at a positive constant value beyond r_b , as shown in Fig. 1. The potentials for the initial and final states are identical for $R \leq r_b$ and the corresponding wave functions are orthogonal to a high degree of accuracy. The decay process arises through the perturbation which lowers the constant potential to its correct value, thus restoring the barrier and allowing the α particle to escape.

Polonium to lead decay

Calculations have been carried out for decays of even polonium isotopes to isotopes of lead, for which the lifetimes and α -particle kinetic energies are well known. For the $O^+ \rightarrow O^+$, $L = 0$, ground state to ground state transitions, equation (2) reduces to

$$\Gamma_{\text{ob}} = 2\pi \left| \int_{r_b}^{\infty} u_0(R)[U_{\alpha A}(R) - U_{\alpha A}(r_b)]\bar{u}_0(R)dR \right|^2 \quad (3)$$

where $\bar{u}_0$, u_0 are respectively the $L = 0$ bound and scattering wave functions of the α particle whose asymptotic forms are

$$\bar{u}_0(R) \rightarrow e^{-\gamma R}, \quad \gamma^2 = \frac{2\mu}{\hbar^2} [\epsilon_{\alpha A}] = \frac{2\mu}{\hbar^2} [U_{\alpha A}(r_b) - T_{\alpha}] \quad (4)$$

$$u_0(R) \rightarrow \sin(kR - n \log 2kR + \eta_0), \quad k^2 = \frac{2\mu}{\hbar^2} T_{\alpha} \quad (5)$$

and $U_{\alpha A}$ is the sum of the nuclear and Coulomb interactions between the α particle and the residual nucleus A. Although there is some latitude in the description of the nuclear interaction because the basic α -nucleon interaction is not uniquely determined, this leads to a variation of only 20–40% in the spectroscopic factor, as indicated in Table 1. Thus the removal of the ambiguity in the potential and the arbitrary radius parameter R_c has eliminated most of the uncertainty in the calculation of α -decay rates, and more precise definition of the barrier height by other experiments would reduce this still further⁹.

Before examining the nuclear structure information represented by the spectroscopic factors we deduce the general relationship between lifetimes and α -particle energies predicted by this theory. In order to obtain such a relationship we neglect the nuclear potential beyond r_b so that

$$U_{\alpha A}(R) - U_{\alpha A}(r_b) = 2Ze^2 \left(\frac{1}{R} - \frac{1}{r_b} \right), \quad R \geq r_b \quad (6)$$

Table 1 Calculated values for decay widths and spectroscopic factors of $L = 0$ decays in polonium isotopes.

Isotope	T_{α} (MeV)	Potential*	Γ_{ob} (MeV)	$S_{\alpha} = \Gamma_{\text{exp}}/\Gamma_{\text{ob}}$
^{210}Po	5.30	H	1.30×10^{-27}	0.029
		H'	1.64×10^{-27}	0.023
^{212}Po	8.80	H	1.02×10^{-14}	0.149
		H'	1.46×10^{-14}	0.104
^{214}Po	7.70	H	5.40×10^{-17}	0.053
		H'	6.80×10^{-17}	0.042

*The symbols H and H' represent different nuclear potentials.

The asymptotic form of $u(R)$ given by equation (5) is likely to be appropriate beyond the outer turning point r_t , which for $L = 0$ is given by $r_t = 2Ze^2/T_a$. Between r_b and r_t the WKB approximation can be used to give¹²

$$u^{(1)}(R) \sim A[K(R)]^{-1/2} \exp\left\{i \int^{r_t} K(r) dr\right\} \quad (7)$$

with $iK(r) = \beta(r)$, where

$$\beta^2(r) = \frac{2\mu}{\hbar^2} \left[U_{\alpha\alpha}(r) - T_a \right], \quad \beta^2(r_b) = \gamma^2, \quad \beta^2(r_t) = 0 \quad (8)$$

Since β^2 is slowly varying, we further approximate the scattering wave function to

$$u^{(2)}(R) \sim e^{B(R)R} \quad (9)$$

so that expression (3) becomes

$$\Gamma \propto \left| \int_{r_b}^{r_t} e^{-\gamma R} \left(\frac{2Ze^2}{R} - \frac{2Ze^2}{r_b} \right) e^{B(R)R} dR + \int_{r_t}^{\infty} e^{-\gamma R} \left(\frac{2Ze^2}{R} - \frac{2Ze^2}{r_b} \right) \sin(kR - n \log 2kR + \eta_0) dR \right|^2 \quad (10)$$

Neglecting the second integral and retaining only the leading term of the first integral, we obtain the approximate expression for the half life T_1 ,

$$\log_e T_1 \propto \{A(T_a) + \log_e B(T_a)\} \quad (11)$$

where, with $x = 2Ze^2/r_b$

$$A(T_a) = 2\gamma r_t = \frac{2Ze^2}{T_a} \left(\frac{2\mu x}{\hbar^2} \right)^{1/2} \left(1 - \frac{T_a}{x} \right)^{1/2} \quad (12)$$

Fig. 2 Plot of $\log_{10} T_1$ for the experimental half lives of even polonium isotopes against $A(T_a)$ (dashed line) and against $A(T_a) + \log_e B(T_a)$ (full line). A value of $r_b = 10.8$ fm predicted by potential H has been used in the calculation.

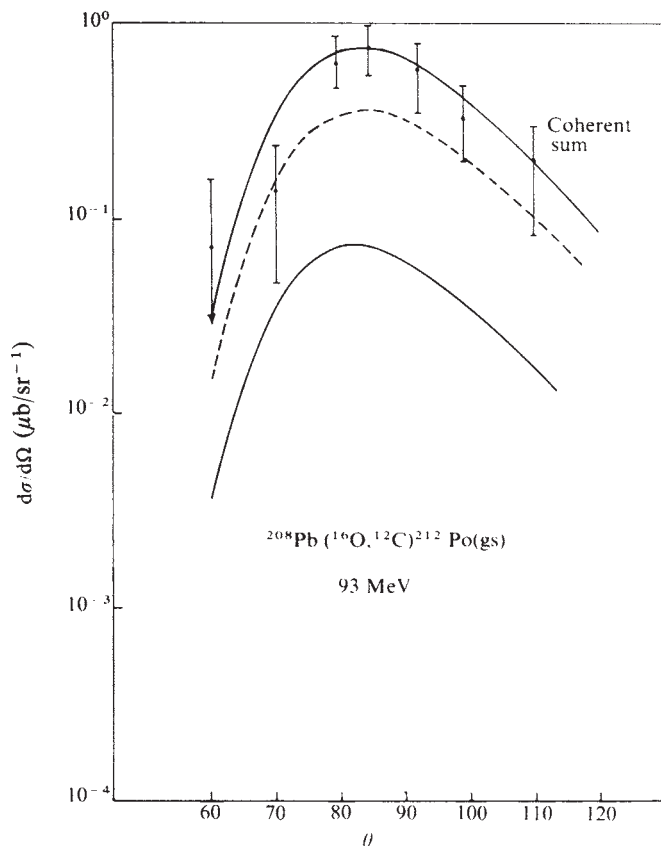
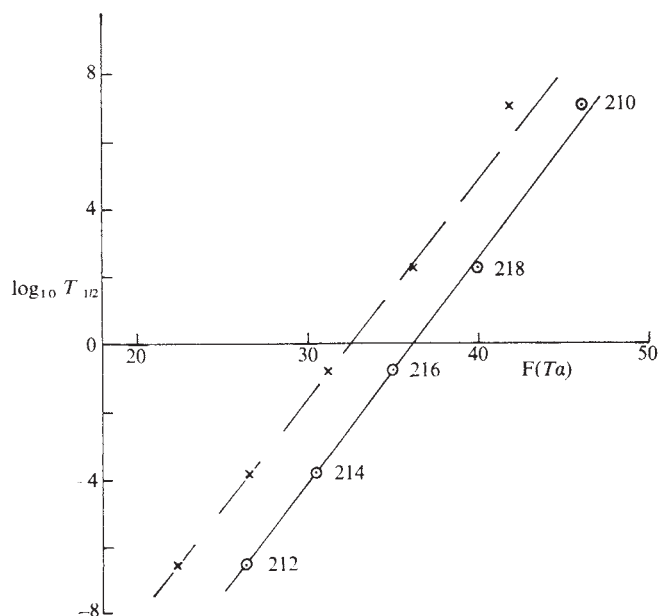


Fig. 3 The angular distribution for the α -transfer reaction $^{208}\text{Pb}(^{16}\text{O}, ^{12}\text{C})^{212}\text{Po}$. The solid line, normalised to the data, represents the coherent sum of the normal DWBA contribution (the dotted line) and an additional contribution arising from our treatment of the α -decaying state (the other solid line).

$$B(T_a) = (\gamma r_b)^2 \left\{ 1 - \frac{r_b}{r_t} \right\}^{-2} = r_b^2 \left(\frac{2\mu x}{\hbar^2} \right) \left(1 - \frac{T_a}{x} \right)^{-1} \quad (13)$$

Although this dependence on T_a does not resemble any of the simple, empirical formulae previously given (see, for example, ref. 13), it can be seen from Fig. 2 that equation (11) does represent the behaviour of the observed half lives of the even isotopes of polonium which cover 14 orders of magnitude, despite the extreme approximations which have been made to derive it. We may reasonably expect that exact calculations using equation (2) should yield the correct lifetime-energy relationship in a wide range of circumstances.

For the particular case of the α decay of the ground state of ^{212}Po to the ground state of ^{208}Pb , which has been widely studied, we obtain spectroscopic factors S_α in the range 0.10–0.15. These values are in excess of the predictions of the shell model (A. Arima and I. Tono-zuka, personal communication) by at least two orders of magnitude. It therefore seems that the shell model is inadequate for this nucleus. The most extreme cluster model in which the cluster expansion is reduced to a single term would yield a spectroscopic factor of unity. The small value of S_α obtained for ^{210}Po reflects the need to break the neutron closed shell to remove an α particle, and here the shell model concept is important.

Checking the results

In order to test our results against different data, we have just completed¹⁴ a study of the reaction $^{208}\text{Pb} + ^{16}\text{O} \rightarrow ^{12}\text{C} + ^{212}\text{Po}$ (gs). In this process an α particle is transferred into ^{208}Pb and it is therefore the inverse of the α -decay process. The initial and final states of the heavy nuclei were described in exactly the same way as in the α -decay calculation. The ^{16}O projectile was

described by the model of Kurath¹⁵ which yields a spectroscopic factor of 0.23 for $^{16}\text{O} \rightarrow ^{12}\text{C} + \alpha$. The spectroscopic factor for $^{212}\text{Po} \rightarrow ^{208}\text{Pb} + \alpha$ was then found by making a distorted wave fit to the angular distribution¹⁶ for the transfer reaction. As can be seen from Fig. 3, the fit to the data is good; it yields a spectroscopic factor of 0.14–0.21 which is in good agreement with the value obtained from α decay.

An alternative means of checking the predicted spectroscopic factors is to calculate them directly using the same nuclear model. We have done this for the 18^+ isomeric state in ^{212}Po which decays to the 0^+ (gs), 3^- and 5^- states in ^{208}Pb . Proceeding as before we find that the spectroscopic factor for the decay to the ground state is 0.13 while a simple perturbation calculation gives the parentage of this stage in the cluster model expansion as 0.25. This is satisfactory agreement given the simplicity of the cluster calculation. The ratio of our calculated one-body widths for the decay of ^{212m}Po and ^{212}Po to the ground state of ^{208}Pb is in very close agreement with experiment where, in contrast, shell model calculations using R-matrix theory¹⁷ are greater than experiment by up to a factor of 45, depending on the channel radius. For the transitions to the lowest 3^- and 5^- states we included in our expansion of the initial state only the 0^+ , 3^- and 5^- states of the residual nucleus. The excitation potentials were parametrised directly in terms of the derivatives of the optical potential with the appropriate transition strength taken directly from inelastic α -particle scattering¹⁸. The encouraging results for this calculation, in contrast to the shell model calculations with varying degrees of configuration mixing¹⁷, suggest that what is important in the decay of the isomeric state is not the collective excitation of the residual

nucleus but rather the cluster structure of the decaying state ^{212m}Po .

All the calculations reported here have been restricted to the expected dominant terms in the cluster model expansion but the formalism will allow the study of many additional effects. Our results also suggest that the very complicated many-body structure of the initial state and the formation of the α -particle are more accurately represented by a cluster model expansion. So far, familiar decay processes have been chosen for study in order to eliminate arbitrary parameters, but there is sufficient information on α -particle decay energies and potential energy surfaces available for superheavy nuclei to make possible a much more realistic estimate of α -decay lifetimes in superheavy nuclei.

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- ¹ Gamow, G. Z. *Phys.* **51**, 204 (1928).
- ² Rutherford, E. *Proc. R. Soc. A* **123**, 373 (1929).
- ³ Mang, H. J. A. *Rev. Nucl. Sci.* **14**, 1 (1964).
- ⁴ Gentry, R. V., Cahill, T., Fletcher, N. R., Kaufmann, H. C., Medster, L. R., Nelson, J. W. & Flocchini, R. G. *Phys. Rev. Lett.* **37**, 629 (1976).
- ⁵ Buttke, P. J. A. *Phys. Lett.* **64B**, 267 (1976).
- ⁶ Moller, P. & Nix, J. R. *Phys. Rev. Lett.* **37**, 1461 (1976).
- ⁷ Scherk, L. & Vogt, E. W. *Can. J. Phys.* **46**, 1119 (1968).
- ⁸ Bencze, G. & Sandulescu, A. *Phys. Lett.* **22**, 473 (1966).
- ⁹ Jackson, D. F. & Rhoades-Brown, M. *Ann. Phys.* (in the press).
- ¹⁰ Harada, K. & Rauscher, E. A. *Phys. Rev.* **169**, 818 (1968).
- ¹¹ DeVries, R. M., Lilley, J. S. & Franey, M. A. *Phys. Rev. Lett.* **37**, 481 (1976).
- ¹² McCarthy, I. E. *Introduction to Nuclear Theory* ch. 2 (J. Wiley, 1968).
- ¹³ Marmier, P. & Sheldon, E. *Physics of Nuclei and Particles* Vol. 1, chapter 7 (Academic Press, 1969).
- ¹⁴ Jackson, D. F. & Rhoades-Brown, M. *Nucl. Phys.* (in the press).
- ¹⁵ Kurath, D. *Phys. Rev.* **C7**, 1390 (1973).
- ¹⁶ Davies, W. G., DeVries, R. M., Ball, G. C., Forster, J. S., McLatchie, W., Shapira, D., Toke, J. & Warner, R. E. *Nucl. Phys.* **A269**, 477 (1976).
- ¹⁷ Rauscher, E. A., Rasmussen, J. O. & Harada, K. *Nucl. Phys.* **A94**, 33 (1967).
- ¹⁸ Alster, J. *Phys. Lett.* **25B**, 459 (1967).

letters to nature

Two new D2 QSOs

MOST extragalactic radio sources fall into one of two categories. In one the emission is extended, with two main peaks tens or hundreds of kiloparsecs apart straddling the associated optical object, while in the other the source is compact (typically less than a kiloparsec in size), with optical and radio positions that coincide to within the errors of measurement. More rarely, double sources may belong to a third category, which was classified by Miley¹ as D2, and of which the type example is 3C273. Sources of this class consist of a small variable nuclear component coincident with the optical object, and a second, larger component with a much steeper spectrum. We report here high resolution observations made with radio-link interferometers based on Jodrell Bank which show that the QSOs 3C345 and 3C454.3 have extended radio structure similar to that of 3C273. Hence these sources, previously believed to be compact, should also be classified as D2. Details of the observations are given in Table 1.

Observations at high frequencies^{2,3} with a resolution of a few arc s detect only compact nuclear components in 3C345 and 3C454.3. Our observations show that the nuclear component of 3C345 is unresolved, with an angular size $\theta < 0.1$ arc s, but suggest that the nuclear component of 3C454.3 is extended, with $\theta \sim 0.5$ arc s in position angle $130 \pm 5^\circ$. High resolution VLBI observations⁴ detect fine structure on a scale of about 10^{-3} arc s in both nuclei. The positions of the nuclear components from our observations³ agree well with the radio positions by Elsmore and Ryle⁵,

and by Rogers *et al.*⁶, and with the optical positions of the QSOs⁷.

In addition to the nuclear components, our observations reveal extended emission from a steep-spectrum companion component in each source. The variations with hour angle of the interferometer fringe amplitude for each baseline listed in Table 1 have been fitted by models which assume components with elliptical Gaussian profiles. The component separations are well determined and do not vary with frequency. The sizes of the individual components themselves, however, are less certain. The best fitting model for the structure of each source is shown schematically in Fig. 1, with details given in the legend. The angular separation of the components corresponds to a projected separation at the source of 14 kpc for 3C345, and 39 kpc for 3C454.3, assuming a Friedmann cosmology with $q_0 = +1$ and $H = 50 \text{ km s}^{-1} \text{ Mpc}^{-1}$. The corresponding value for 3C273 is 70 kpc.

Table 1 Observations

Frequency (MHz)	Interferometer baseline (km)	Minimum lobe size (arc s)	Epoch of observations	
			3C345	3C454.3
408	24	6.3	1969.9 (ref. 16), 1971.7, 1972.7, 1971.7	
408	127	1.2	1970.9 (ref. 17)	1974.7, 1975.9
962	24	2.7	1973.9	1973.9
1,666	24	1.6	1973.3	1973.3
1,666	127	0.3	1974.8 (ref. 18)	

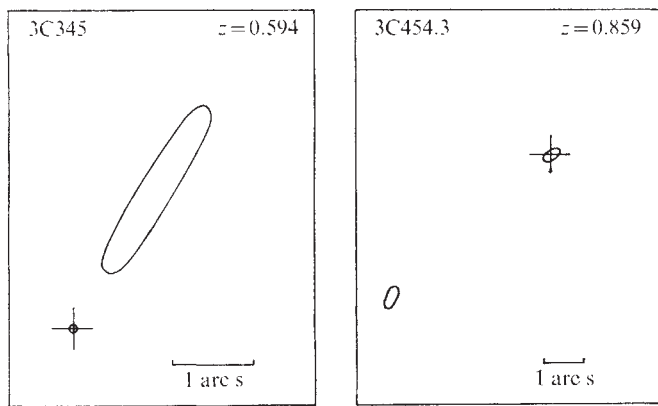


Fig. 1 The radio structures of 3C345 and 3C454.3. North is to the top, increasing Right Ascension to the left. In each source the nuclear component is distinguished by a cross. The components of 3C345 have a separation of 2.1 ± 0.1 arc s in p.a. $149 \pm 6^\circ$. The nuclear component is smaller than 0.1 arc s in all directions; the companion component has a size of $\sim 2.5 \times 0.5$ arc s and is elongated in p.a. $142 \pm 6^\circ$. The components of 3C454.3 have a separation of 5.4 ± 0.1 arc s in p.a. $131 \pm 1^\circ$. The nuclear component has a size of $\sim 0.5 \times 0.3$ arc s and is elongated in p.a. $130 \pm 5^\circ$; the companion component has a size of 0.7×0.3 arc s and is elongated in p.a. $165 \pm 10^\circ$.

Marked variations have been reported in the flux density of 3C454.3 at frequencies below 1 GHz (refs 8, 9). The 408-MHz flux density was 14.0 ± 0.7 Jy at epochs 1971.7 and 1972.7, but had risen to 15.9 ± 0.7 Jy in 1974.7, and was 14.8 ± 0.7 Jy in 1975.9 (flux densities on the KPW scale¹⁰). The fringe amplitude data indicate that it is the nuclear component alone which is responsible for these variations. Our measurements of 3C345 are less extensive but suggest that neither component of this source varied by more than 10% in the 2-yr period of our observations at 408 MHz.

The flux densities of the components of 3C345 and 3C454.3 are shown in Fig. 2 together with the integrated spectrum^{11,12} of each source. Both nuclear components have slightly inverted spectra in the range 408–1,666 MHz, whereas the companion components have steep spectra, with spectral indices -1.4 ± 0.2 and -1.0 ± 0.1 respectively. It is clear from Fig. 2 that the rise in flux density at the low-frequency end of the integrated spectrum may be attributed to the companion component in each case. It may well be that other QSOs with D2 structure could be recognised from the shape of their integrated spectra.

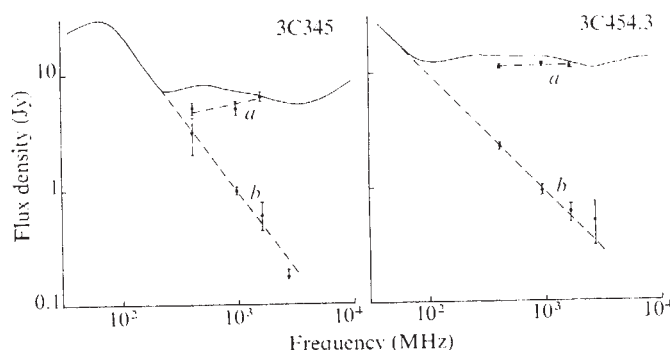


Fig. 2 The radio spectra of 3C345 and 3C454.3. The continuous curves are the integrated spectra from refs 11 and 12. The spectra of the nuclear and companion components are labelled *a* and *b* respectively. The points at 2,695 MHz have been obtained by re-interpreting the visibility data of Bash² in terms of our model structures.

It is interesting to note that 3C273, 345 and 454.3 are all optically violently variable¹³. In the case of 3C273 the companion radio component is associated with a faint optical jet^{14,15}. By analogy, it may be worthwhile to search for optical counterparts to the companion radio components of 3C345 and 3C454.3. There are however considerable practical difficulties in detecting faint optical jets only a few arc s away from ~ 16 mag QSO nuclei.

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- ¹ Miley, G. K. *Mon. Not. R. astr. Soc.* **152**, 477–490 (1971).
- ² Bash, F. N. *Astrophys. J. Suppl.* **16**, 373–404 (1968).
- ³ Elsmore, B. & Ryle, M. *Mon. Not. R. astr. Soc.* **174**, 411–423 (1976).
- ⁴ Wittels, J. J. *et al. Astrophys. J.* **196**, 13–39 (1975).
- ⁵ Warwick, R. S., Davis, R. J. & Spencer, R. E. *S. Mon. Not. R. astr. Soc.* **177**, 335–347 (1976).
- ⁶ Rogers, A. E. E. *et al. Astrophys. J.* **186**, 801–806 (1973).
- ⁷ Argue, A. N. & Kenworthy, C. M. *Mon. Not. R. astr. Soc.*, **160**, 197–211 (1972).
- ⁸ Hunstead, R. W. *Astrophys. Lett.* **12**, 193–200 (1972).
- ⁹ Hayes, P., Conway, R. G. & Stannard, D. *Mon. Not. R. astr. Soc.* **169**, 117–131 (1974).
- ¹⁰ Kellermann, K. I., Pauliny-Toth, I. I. K. & Williams, P. J. S. *Astrophys. J.* **157**, 1–34 (1969).
- ¹¹ Kellermann, K. I. & Pauliny-Toth, I. I. K. *Astrophys. J.* **155**, L71–78 (1969).
- ¹² Kellermann, K. I. and Pauliny-Toth, I. I. K. *Astrophys. Lett.* **8**, 153–160 (1971).
- ¹³ Penston, M. V. and Cannon, R. D. *R. Obs. Bull.* **159**, 85–109 (1970).
- ¹⁴ Schmidt, M. *Nature* **197**, 1040 (1963).
- ¹⁵ Conway, R. G. & Stannard, D. *Nature*, **255**, 310–312 (1975).
- ¹⁶ Wilkinson, P. N. *Mon. Not. R. astr. Soc.* **160**, 305–319 (1972).
- ¹⁷ Peckham, R. J. *Mon. Not. R. astr. Soc.* **165**, 25–38 (1973).
- ¹⁸ Bentley, M., Hayes, P., Spencer, R. E. & Stannard, D. *Mon. Not. R. astr. Soc.* **173**, 93–97P (1975).

Nova shells: matter-bound or radiation-bound?

THE ionisation structure of nova shells is quite uncertain. Many recent papers^{1–2,11} have assumed that the shell is fully ionised during the decline, and that the rate of decline is a sensor of the matter density in the expanding shell. In this letter we propose that the shell of V1500 Cyg is not fully ionised, but rather forms a Strömgren shell. In this case the optical light curve is determined by the decline of the energy production rate of the central object.

The spectrum of a typical fast nova (V1500 Cyg, CP Pup, CP Lac) during outburst is first characterised by a moderately hot ($\sim A$ type) optically thick photosphere. The expanding material becomes optically thin after maximum^{1,11} and after a certain time, which varies from nova to nova, the spectrum changes to that of an H II region or planetary nebula.

Recent papers have assumed that the expanding shell is completely ionised during this nebular phase. In this case the size of the emitting region is determined only by the spatial extent of the shell: the emitting region is said to be matter-bound. The hydrogen emission from a matter-bound envelope is given by

$$E \propto \int_V N_e N_p \alpha_{\text{ff}}(T) dV + \int_V N_e N_p \alpha_{\text{fb}}(T) dV \quad (1)$$

where the electron and proton densities are particles cm^{-3} and the temperature sensitive free-free and free-bound cross sections have the usual definition. We have neglected the effects of line optical depth.

If the shell is homogeneous and hydrogen is the dominant electron donor then equation (1) simplifies to

$$E \propto N_e^2 V \quad (2)$$

For a completely ionised nebula the electron density is directly proportional to the local matter density so we can write

$$E \propto \rho^2 V \propto V^{-2} V \propto V^{-1} \propto t^{-\beta}$$

where t is time. The expansion law is determined by β ; $\beta = 2$ for a thin shell expansion (linear thickness of the shell is independent of time), and $\beta = 3$ for a Hubble law expansion ($\delta r/r = \text{constant}$). The latter may be more appropriate for an explosion. We see that the emission from a matter-bound shell is determined mainly by the matter density. Actually there is also a temperature dependence because of the temperature-sensitive cross sections.

The time of onset of the expansion of a matter-bound shell may be determined from the light curve². The times of onset derived with this technique are typically about one month before optical maximum.

The size of the photosphere at maximum is a simple test of the plausibility of this model. Using V1500 Cyg as an example and assuming that the main shell was ejected 35 days before maximum² at a velocity of 1,500 km s⁻¹ (ref. 3) we find that the optically thick photosphere would have a radius of over 30 AU at maximum, about ten times larger than observed¹. This hypothesis is also in substantial disagreement with the observed blackbody expansion curves^{1,4}.

In this letter we question the basic assumption that the shell is matter-bound. Following the work of Pottasch⁵ we propose that the shell is radiation-bound, that is, that the volume of the emitting region is determined by the balance between photoionisation and recombinations. The most direct evidence that the V1500 Cyg shell was not fully ionised is the observation³ of neutral oxygen emission long after maximum. This was observed both as [O I] 6300, 6363 (ref. 6) and O I 8446, 7774 (ref. 9).

We shall consider the ratio of flux in H β to that in [O I] 6300. This ratio was ≈ 10 for much of the decline⁶. We shall predict this ratio for a fully ionised shell in the following manner. The H β emission from an optically thin unit volume is

$$E(\text{H}\beta) = h\nu_{\beta} N_e^2 \alpha_{\text{H}\beta}^{\text{eff}} = h\nu_{\beta} N_e^2 3.1 \times 10^{-14} \text{ cm}^3 \text{ s}^{-1} \quad (3)$$

where we have assumed a temperature of 10⁴ K and an effective case B recombination coefficient⁷. An upper limit to the energy in λ 6300 may be estimated by assuming that the ¹D₂ level is populated as in LTE at 10⁴ K. The emission from the thermalised ¹D₂ level will be

$$\begin{aligned} E(6300) &= h\nu_{6300} N(\text{O I}) g(\text{O I}) e^{-h\nu/kT} A(6300) \\ &= h\nu_{6300} N(\text{O I}) 3 \times 10^{-4} \end{aligned} \quad (4)$$

where we have again assumed a temperature of 10⁴ K. The intensity ratio will be

$$\frac{E(\text{H}\beta)}{E(6300)} = \frac{6300}{4861} N_e^2 N(\text{O I})^{-1} 1 \times 10^{-10} \quad (5)$$

We can predict the fractional abundance of O I by assuming that it is coupled to the fractional abundance of H I by charge exchange

$$\frac{N(\text{O I})}{N(\text{O II})} \approx \frac{9}{8} \frac{N(\text{H I})}{N(\text{H II})} = \frac{9}{8} \frac{\xi}{(1-\xi)} \approx \frac{9}{8} \xi \quad (6)$$

where ξ is the fraction of neutral hydrogen and we anticipate our result that hydrogen is highly ionised. An upper limit to

the oxygen emission can be obtained by assuming that all oxygen is in one of these two species.

We can estimate ξ by equating photoionisation with recombinations and assuming steady state. The balance equation at a typical point in the shell is

$$N(\text{H I}) \sigma \frac{Q(\text{H}) e^{-\tau}}{4\pi r^2} = N_e^2 \alpha_{\text{H}}(I) \quad (7)$$

where σ is the target area for photoionisation ($\approx 6 \times 10^{-18} \text{ cm}^2$), $Q(\text{H})$ is the number of ionising photons emitted by the central object, τ is the optical depth to ionising photons (for a matter-bound shell τ is small so we shall take $e^{-\tau} \approx 1$), and r is the radius of the shell. We will evaluate the parameters at a time 30 d after the outburst. Assuming an expansion velocity of 1,500 km s⁻¹ we obtain a radius of $4 \times 10^{14} \text{ cm}$. Unpublished McDonald scans show that H β had a strength of $5 \times 10^{-17} \text{ J s}^{-1} \text{ cm}^{-2}$ which corresponds to 2.6×10^{47} photon s⁻¹ if we assume¹⁰ a colour excess of $E(B-V) = 0 \text{ mag}$. 50 and a distance of 1,800 pc. The assumption that the shell is matter-bound requires that $Q(\text{H})$ be much larger than the photoionisation rate of $2.6 \times 10^{47} \alpha_{\text{B}}/\alpha_{\text{H}\beta}^{\text{eff}} \approx 2.4 \times 10^{48} \text{ s}^{-1}$. Using these parameters we obtain

$$\frac{N(\text{H I})}{N_e^2} = \frac{\alpha_{\text{B}}(T) 4\pi r^2}{\sigma Q(\text{H})} = 6 \times 10^{-14} \text{ cm}^3$$

or

$$\xi = N_e 6 \times 10^{-14} \approx 10^{-5}$$

where we have assumed an electron density of 10⁸ cm⁻³. The ratio of line strengths will be

$$\frac{E(\text{H}\beta)}{E(6300)} = \frac{N(\text{H})}{N(\text{O})} 7.3 \times 10^{-5} N_e \approx 0.02 N_e \approx 10^6$$

where we have included solar abundances⁸. We see that the observed strength of [O I] 6300 is five orders of magnitude stronger than the predicted value for a fully ionised shell. Enhanced oxygen abundances cannot resolve this discrepancy.

The observed strength of O I is easily understood if the shell is radiation-bound. The O I emission originates at the edge of the hydrogen Strömgren sphere where both free electrons and neutral oxygen are abundant.

The presence of an H Strömgren sphere has important consequences. The optical emission from the envelope will be a sensitive indicator of the emission of the central object at frequencies below the H ionisation threshold since the radius of the Strömgren sphere adjusts itself so that the number of photoionisations equals the number of recombinations. Thus we see that published light curves² of nova shells which are optically thin in visible radiation but which are optically thick to ionising photons are valuable indicators of the decay of energy generation of the central object. The cool outer neutral zone may also provide an auspicious site for the dust formation which seems to be a common feature of the nova outburst¹¹.

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¹ Gallagher, J. S. & Ney, E. P. *Astrophys. J.* **204**, L35 (1976).

² Leibowitz, E. M. *Nature* **264**, 41 (1977).

³ Tomkin, J., Woodman, J. & Lambert, D. L. *Astron. Astrophys.* **48**, 319 (1976).

⁴ Barnes, T. G. *MNRAS* **177**, 53p (1976).

⁵ Pottasch, S. *Ann. Astrophysique* **22**, 394 (1959).

⁶ Ferland, G. J., Lambert, D. L. & Woodman, J. *Astrophys. J.* **213**, 132 (1977).

⁷ Osterbrock, D. E. *Astrophysics of Gaseous Nebulae*, (Freeman and Co., 1974).

- ⁸ Cameron, A. *Explosive Nucleosynthesis*, (eds Schramm and Arnett) (University of Texas Press, 1973).
⁹ Ferland, G. J. & Wootten, H. A. *Astrophys. J.* 214, L27 (1977).
¹⁰ Ferland, G. J. *Astrophys. J.* (in the press).
¹¹ Ennis, D., Becklin, E., Beckwith, S., Elias, J., Gatley, I., Matthews, K., Neugebauer, G. & Willner, S. (in the press).

Effect of electrojet on the total electron content of the ionosphere over the Indian subcontinent

THE radio beacon method of measuring TEC along a chain of optimally spaced stations near the equatorial anomaly has been used to monitor continuously the latitudinal extent of the anomaly. Comparisons with the magnitude of the electrojet show a clear dependence of anomaly strength upon the electrojet intensity with an approximate two-hour time delay.

Anomalous latitudinal distribution of electron density in the F2 region, often referred to as the equatorial anomaly, was found by Appleton¹. The diurnal development of the equatorial anomaly has been studied by Rastogi². Dunford³ showed that the equatorial anomaly in the topside ionosphere is correlated with the E region current system near the magnetic equator. With the development of the satellite radio beacon technique numerous workers⁴⁻⁹ studied the Appleton anomaly in total electron content (TEC) of the ionosphere with greater spatial resolution, but with poor temporal resolution, as only a few passes per day of the low orbit satellite could be received. Iyer *et al.*¹⁰ showed that the equatorial anomaly in TEC over India is positively correlated with the electrojet strength. The diurnal development of the equatorial anomaly in TEC is presented here for the first time; and, along with magnetometer records which indicate the strength of the equatorial electrojet, the TEC anomaly is shown for three days in May 1976. The equatorial anomaly in TEC was continuously monitored over the Indian subcontinent using radio beacon signals from the geostationary satellite ATS-6, received at many locations in India, namely: Bombay (19.8°N, 69.8°E), Rajkot (20.8°N, 67.7°E), Ahmedabad (21.5°N, 69.4°E), Udaipur (22.9°N, 70.2°E), Jaipur (25.1°N, 71.9°E), and Patiala (28.2°N, 72.1°E). Coordinates given are 350-km intersections along the ray path to the ATS-6 satellite. Details of the stations in the Indian subcontinent measuring TEC using ATS-6 beacon transmissions have been given by Iyer *et al.*¹¹.

The diurnal development of the equatorial anomaly in TEC and its association with electrojet strength are clearly shown in Fig. 1. Three typical cases are chosen: (1) a normal electrojet day, 18 May 1976, $A_p=3$; (2) a counter electrojet day, where the counter electrojet occurred in early afternoon, 12 May 1976, $A_p=7$, and (3) a geomagnetically disturbed day, 3 May 1976, $A_p=94$. For all three days the horizontal component of the magnetic field (H) for an equatorial station, Kodaikanal, dip 3.4°N, are also shown. The magnetic field during daytime is a representative indication of the electrojet strength. In addition TEC plots against latitude are shown for different local times of the day. On a normal electrojet day the magnetic field shows a flat minimum night value (Fig. 1a). After sunrise the electron density in the E region increases and the H field shows a steady increase until around 1100 h, after which it starts decreasing. Such behaviour of the magnetic field is due to the eastward electric field (E) during daytime. Owing to this electric field and the north-south magnetic field at the equator $E \times B$ drifts are produced and the electrons are lifted to higher altitudes. The lifting of electrons takes place up to a few scale heights above the F2 region peak, beyond which they diffuse along the lines of force and are deposited in the latitude regions of about 15° to 20° dip latitude. This process takes approximately

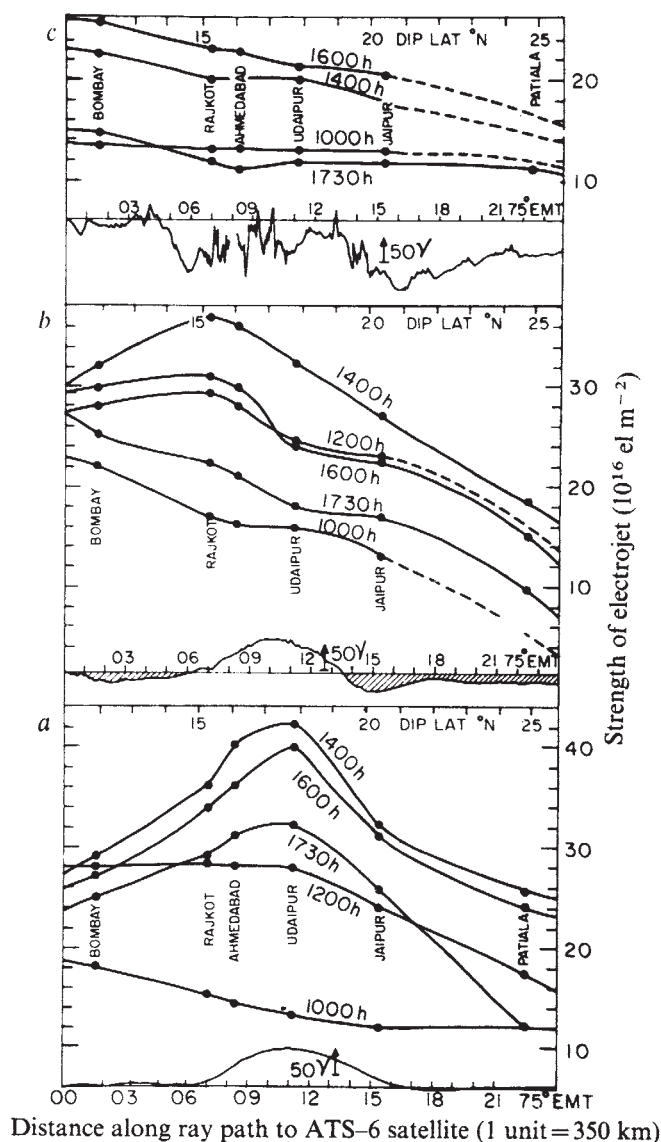


Fig. 1 Diurnal development of equatorial anomaly and strength of electrojet. *a*, Normal electrojet day. Note that the anomaly is fully developed during afternoon hours. *b*, Counter-electrojet day, where anomaly is reduced during afternoon hours. *c*, Disturbed day, where anomaly is not at all developed.

2 h (ref. 10) and any effects in electric fields at E region heights at the equator are seen at latitudes of 15° to 20° after an approximate two-hour delay. At 1000 h, the latitudinal profile of TEC is showing a normal behaviour with high equatorial values (Fig. 1a). As time progresses, at 1200 h, a weak anomaly develops with maximum TEC at Udaipur. By 1400 h more electrons have flowed along field lines to Udaipur and the anomaly is well developed. The process continues until evening and a clear maximum is still seen at 1730 h at Udaipur on this normal electrojet day.

The H component of the equatorial magnetic field shows the start of a normal electrojet in the forenoon hours (Fig. 1b). However, at approximately 1340 h the direction of the H component reverses and a counter electrojet has begun. The latitudinal profiles of TEC for several times on 12 May show a small latitudinal anomaly at 1200 h, reaching a maximum at 1400 h, at the Rajkot ionospheric intersection point (Fig. 1b). But, by 1600 h, and later at 1730 h, there is little, if any, trace of the anomalous latitudinal behaviour in TEC, due to the counter electrojet which developed in early afternoon. The reversal of the

electric field causes downward drift motion at the equator and the movement of ionisation to the latitude range of 15–20° becomes ineffective. The approximate two-hour delay between the start of the counter electrojet and the near-end of the latitudinal TEC anomaly is clearly seen in Fig. 1b.

Data for a highly geomagnetically disturbed day, 3 May 1976, are also shown (Fig. 1c). On this day the electrojet does not occur and the transfer of ionisation from the equator to higher latitudes does not take place to any noticeable extent.

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- 1 Appleton, E. V. *J. atmos. terr. Phys.* **5**, 348 (1954).
- 2 Rastogi, R. G. *J. geophys. Res.* **64**, 727 (1959).
- 3 Dunford, E. J. *J. atmos. terr. Phys.* **29**, 1489 (1967).
- 4 Basu, S. & Das Gupta, A. *J. Geophys. Res.* **73**, 5599 (1968).
- 5 Titheridge, J. E. & Smith, W. D. *Planet. Space Sci.* **17**, 1667 (1969).
- 6 Mendonca, F. de, Cantor, I. J. & Clemesha, B. *Low Latitude Ionospheric Electron Content Measurements During Half a Solar Cycle Relat. Cient. LAFE-84 Lab de Fisica, Espacial Sao Jose dos Campos, Brazil* (1969).
- 7 Golton, E. & Walker, G. O. *J. atmos. terr. Phys.* **33**, 1 (1971).
- 8 Rastogi, R. G., Sharma, R. P. & Shodhan, V. *Planet. Space Sci.* **21**, 713 (1973).
- 9 Rastogi, R. G. & Rajaram, G. *Ind. J. Pure appl. Phys.* **9**, 531 (1971).
- 10 Iyer, K. N., Deshpande, M. R. & Rastogi, R. G. *Proc. Ind. Acad. Sci.* **84**, 129 (1976).
- 11 Iyer, K. N., Deshpande, M. R., Sethia, G. & Rastogi, R. G. *Proceedings of the Workshop on Beacon Satellite Studies*, Physical Research Laboratory, Ahmedabad, pp. 64–86, Dec. 1976.

Hydrothermal plumes in the Galapagos Rift

ALTHOUGH there is indirect evidence that a major fraction of the heat loss from newly-created lithosphere occurs by convection of seawater through the porous crust^{1–3}, it has proved difficult to locate vents of deep-sea hydrothermal systems by direct measurement of the discharge fluid. Local increases in bottom water temperature up to 0.1 °C have been measured by towing arrays of thermistors a few metres above the axes of active oceanic spreading centres^{4,5}, but these data are ambiguous because small temperature anomalies may have a hydrographic explanation. We report here the first conclusive measurements of modified seawater discharging as buoyant hydrothermal plumes from fissures in young oceanic crust. We obtained samples of hydrothermal plumes in the Galapagos Rift³, albeit after considerable dilution with surrounding bottom-water, and report the first results of the collection and analysis of these samples.

Our initial estimates of properties useful for detecting deep-ocean hydrothermal fluids were based on the geochemistry of the Red Sea brines^{6,7}, the only oceanic geothermal system previously sampled. In this system the basalt-interaction imprint is unfortunately superimposed on a saturated brine because the seawater flows through evaporite deposits before entering the basalts⁸. Predicted concentrations for constituents masked by the evaporite contribution must therefore be based on laboratory measurements⁸. Our estimates showed that helium isotopes were by far the most sensitive geochemical tracers: a mixture of one part of hydrothermal fluid in ~10⁵ parts of seawater should be detectable in the ³He/⁴He isotope ratio, while a dilution of ~2×10⁴ should be detectable with total helium concentration. The latter figure is comparable with the detection limit for temperature differences, assuming a 50 °C fluid temperature. Transition metals such as Mn and Fe are also highly enriched in the Red Sea brines but the observed enrichments are consistent with an origin from evaporites⁹ and ³He is the only tracer which cannot be derived from such a source⁷.

During the May 1976 Pleiades Expedition of the Scripps Institution, near-bottom hydrographic and geochemical surveys of sites on the East Pacific Rise (4°S, 102°W) and in the Galapagos Rift (1°N, 86°W) were made with a remote sensing and sampling system attached to the deep tow geophysical vehicle of the Scripps Marine Physical Laboratory¹⁰. The geochemical system included a high-precision CTD instrument to measure conductivity, temperature, and pressure, a light transmissometer, and an array of 8 remotely-triggered 9-litre sampling bottles designed for rapid flushing. The data were telemetered to a shipboard computer which continuously monitored depth, potential temperature (θ), salinity (S), potential density at 4,000 decibars (σ_t), and light transmission in real time. Measurement precision was ± 1 m in depth, ± 0.0015 °C in θ , and ± 0.002 ‰ in S . At each site the instrument package was towed ~8–100 m above bottom at speeds of ~1–2 knots (30–60 m min⁻¹) for about four days, using acoustic transponders for navigation and sonars and cameras for mapping bottom topography.

Near-bottom potential temperature excursions or 'spikes' were encountered in both survey areas; two examples are shown in Fig. 1 (upper traces). Hydrographic or mixing spikes ('false plumes') were characterised as such in the following way. The temperature–salinity (θ – S) relationship for the local water mass was first established by continuous soundings with the vehicle. The observed θ – S relationship (linear at the spreading axis depth) was then programmed into the computer and the appropriate function of θ , S which is invariant to mixing was monitored continuously. Deviations from this relationship were assumed to represent possible hydrothermal plumes and sampling was triggered manually when such deviations were observed.

Fig. 2 shows θ – S data points collected over several hours of towing during the East Pacific Rise survey. Bottom water temperatures west of the rise in this area are ~0.1 °C lower than at comparable depths east of the rise, while at the crest itself there is a transition region which is actually a sharp benthic thermocline (Fig. 2a). Fig. 2b, which contains all the rise-crest data from Fig. 2a, shows that only a single water mass, characterised by a linear θ – S relationship, is present over the entire region. The transition region at the rise crest thus simply reflects linear mixing between the water types on either side of the crest; as the deep tow vehicle navigates through the rough topography the θ record exhibits spikes caused by vertical excursions of the sensor and by encounters with various members of the continuous array of water types available in eddies and streams on the crest. Heated bottom water, however, will deviate from the ambient

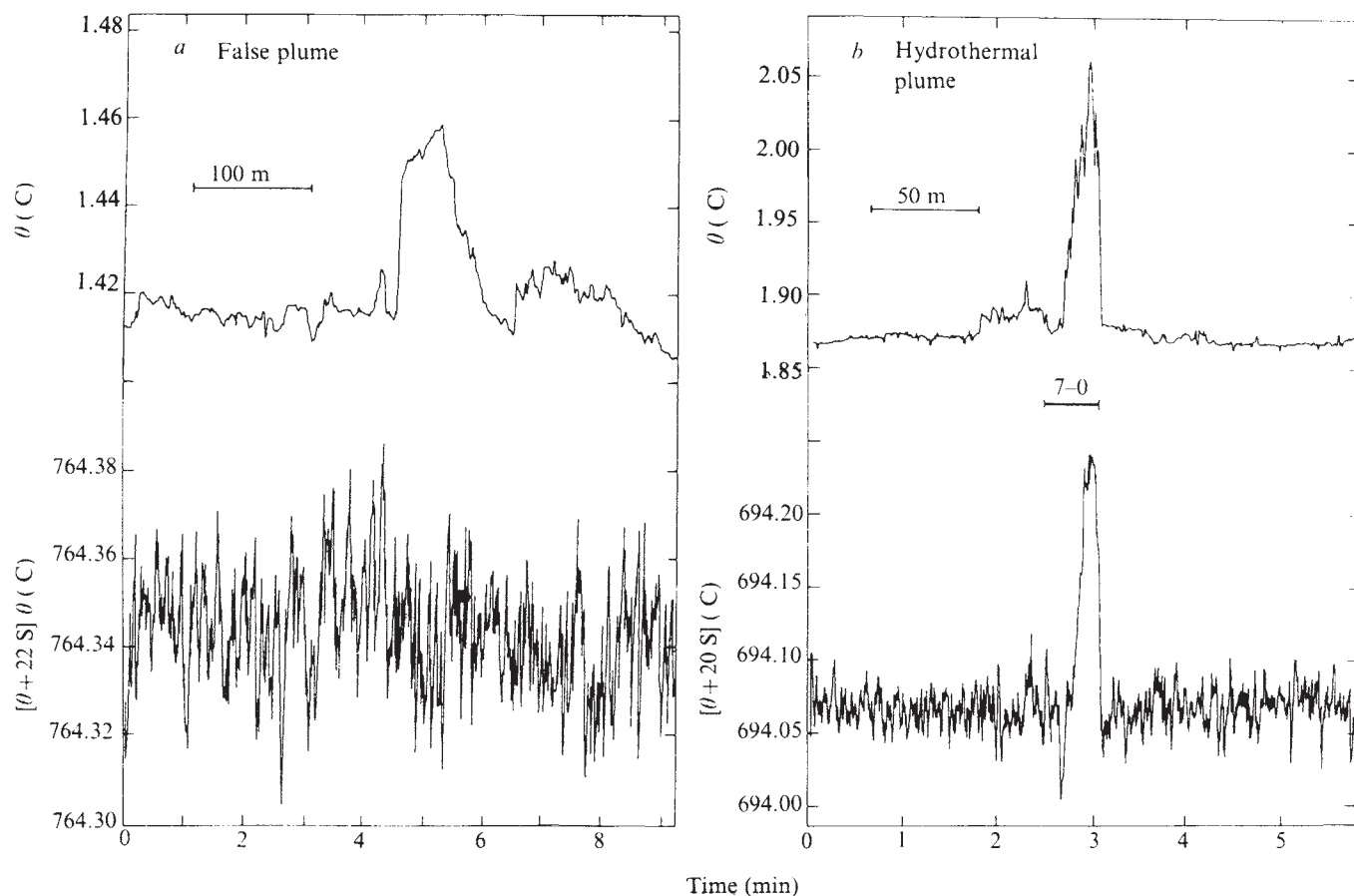


Fig. 1 Temperature records from an arbitrary time zero as the deep tow vehicle is towed ~ 10 m above bottom. Upper traces: potential temperature (θ) plotted at 10 points s^{-1} , corrected for a 200-ms time constant and smoothed by 10-point running average. Lower traces: the θ -salinity function ($\theta + X S$), which is invariant to mixing, with similar correction and smoothing. *a*. A record taken 1.9 km west of the East Pacific Rise axis, shows a 'false plume' in which the temperature spike is not seen in the θ - S function and is thus due to mixing. Tow speed was 50 m min^{-1} . *b*. The Galapagos Rift site data show a true hydrothermal spike which is reflected in both the temperature and $\theta + X S$ records. Tow speed was 43 m min^{-1} and sample 7-0 was collected during the interval shown beneath the θ record.

θ - S relationship characterised by $\theta + X S = \text{constant}$, where X is the negative slope on the θ - S plot (Fig. 2b). Because of the possibility of conductive heating, the detection of such a ' θ - S spike' is a necessary, but not sufficient, con-

dition for the existence of an actual hydrothermal plume.

The lower trace in Fig. 1a is the real time record of the θ - S function measured concurrently with the East Pacific Rise θ record shown above, and plotted to the same

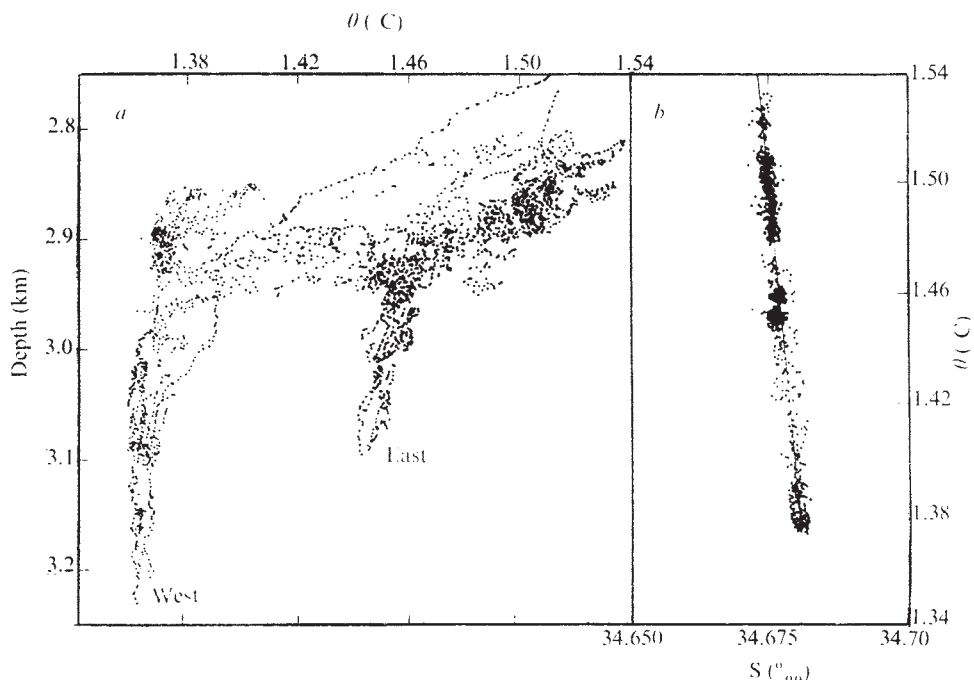


Fig. 2 Near-bottom (10-100 m) potential temperature data points measured over several hours of towing at the East Pacific Rise survey site, plotted against depth (Fig. 2a) and salinity (Fig. 2b). The θ - S diagram (2b) shows that the entire array of water types represents a single water mass; the θ - S slope here ($= -X$) is -22 .

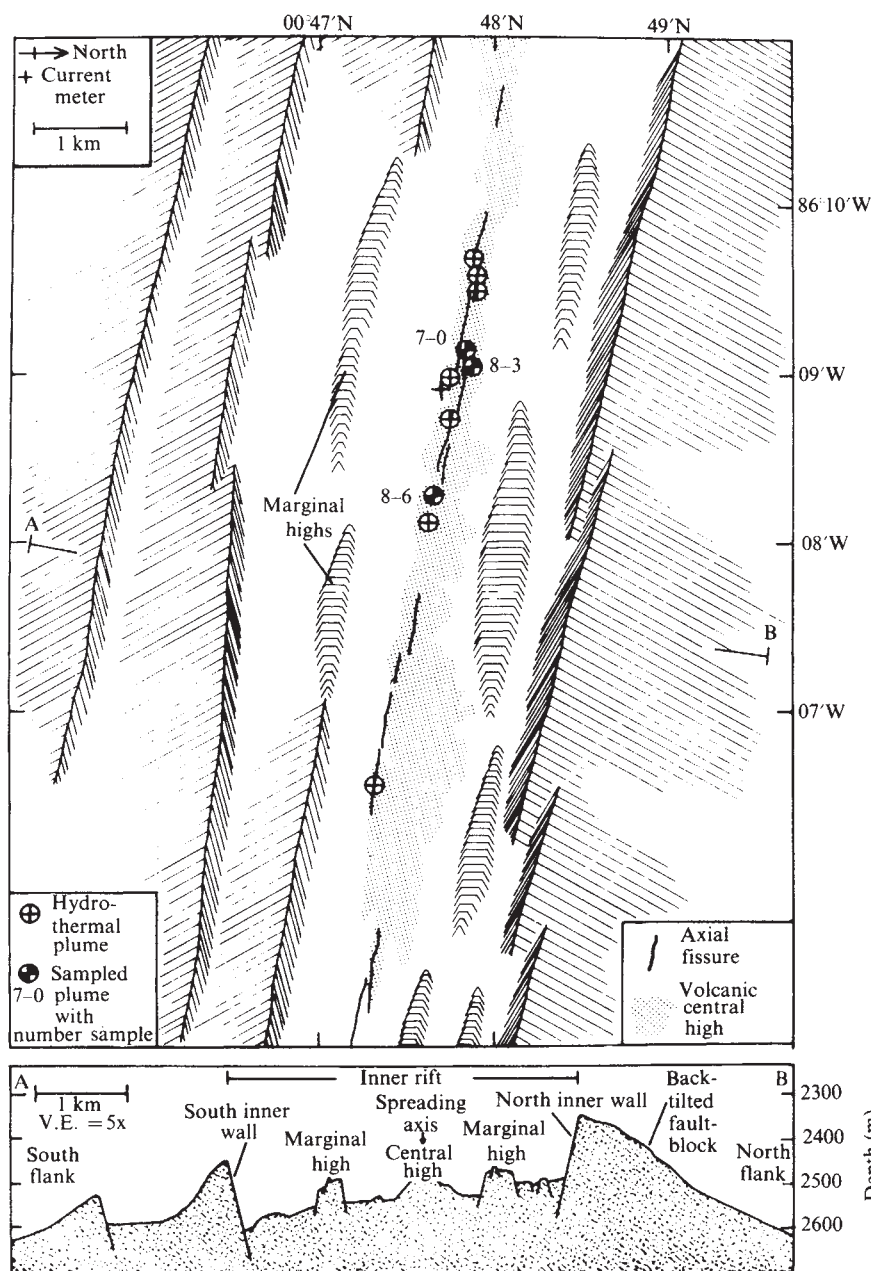


Fig. 3 Locations of hydrothermal plumes observed in the Galapagos Rift, with topography summarised from a deep tow structural map¹⁴. Numbers refer to plumes actually sampled. In addition to the axial fissures shown there are many fissures and minor faults along the minor rift margins; no plumes were detected in these marginal areas.

vertical scale. This spike is clearly due to mixing and is labelled a 'false plume'. It is apparent that a ' θ -S spike' requires an amplitude of $>0.02^\circ$ for detection due to the scatter introduced by the conductivity measurement. Within this limitation, no hydrothermal spikes were observed during the East Pacific Rise survey.

Fig. 1b shows a potential temperature spike measured on the Galapagos Rift which shows a corresponding excursion in the θ -S function. In this area the near-bottom temperature structure reflects mixing between bottom water entering the Panama Basin through the Ecuador Trench¹⁰ and warmer overlying water which spills across the Carnegie Ridge¹¹. The θ -S excursion shows clearly that in this case the θ anomaly is not caused by mixing. Sample 7-0, collected somewhere in the indicated time interval, had a $^3\text{He}/^4\text{He}$ ratio equal to twice the atmospheric ratio, a spectacular anomaly relative to 'background' water which has a ratio only 30% greater than atmospheric¹². This is by far the largest $^3\text{He}/^4\text{He}$ ratio yet observed in deep ocean water and there is thus no

doubt that an actual hydrothermal plume was sampled.

Fig. 3 shows the bathymetry of the Galapagos Rift area as surveyed during the present study. The lava flows in the inner rift valley are fractured by many small faults and fissures along the rift margins, but near the central axis fracturing is confined to a few fissures about two or three metres wide extending along the central high^{13,14}. Ten temperature spikes with corresponding ' θ -S spikes' were observed in this region during the shipboard survey; the locations of these ten spikes are plotted in Fig. 3 which shows that all ten lie on the axial fissure in the centre of the inner rift. Three of these spikes, 7-0 (the largest of the ten spikes) 8-3, and 8-6, were sampled and analysed; all three showed $^3\text{He}/^4\text{He}$ and radon anomalies relative to background water, verifying that the θ -S excursions in these ten locations represent true hydrothermal plumes.

A preliminary analysis of the remaining tow data has shown no evidence of hydrothermal activity off the central axis of the Galapagos Rift. One area of special interest

in our survey was a region of 5–20 m high sediment mounds ~30 km south of the central axis which have been described as possible hydrothermal vents¹³. In this area the water structure is so uniform that hydrothermal spikes of ~0.002 °C should be observable, yet even with this sensitivity there was no evidence of hydrothermal plumes associated with the mounds.

Several of the hydrothermal plumes showed a small decrease (~0.1%) in light transmission; filtrates from the three sampled plumes and water have been distributed for a number of geochemical measurements. The helium and radon measurements are described in an accompanying paper¹². Our results show that there is a one-to-one correspondence between the hydrographic identification of samples as heated water and the presence of excess quantities of ³He/⁴He and Rn in these samples. Together, these results constitute the first conclusive identification and sampling of hydrothermally circulating seawater in a deep-ocean spreading centre. Based on these findings, a detailed study of the Galapagos Rift hydrothermal vents using a manned submersible is now in progress.

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- ¹ Talwani, M., Windisch, C. C. & Langseth, M. G. *J. Geophys. Res.* **76**, 473–517 (1971).
- ² Lister, C. R. B. *Geophys. J.R. astr. Soc.* **26**, 515–535 (1972).
- ³ Sclater, J. G. & Klitgord, K. D. *J. Geophys. Res.* **78**, 6951–6975 (1973).
- ⁴ Williams, D. L., Von Herzen, R. P., Sclater, J. G. & Anderson, R. N. *Geophys. J.R. astr. Soc.* **38**, 587–608 (1974).
- ⁵ Rona, P. A., McGregor, B. A., Betzer, P. R. & Krause, D. C. *Deep-Sea Res.* **22**, 611–618 (1975).
- ⁶ Craig, H. *Hot Brines and Recent Heavy Metal Deposits in the Red Sea* (eds Degens, E. T. & Ross, D. A.) Springer-Verlag, New York, 208–242 (1969).
- ⁷ Lupton, J. E., Weiss, R. F. & Craig, H. *Nature* **266**, 244–246 (1977).
- ⁸ Bischoff, J. L. & Dickson, F. W. *Earth Planet. Sci. Lett.* **25**, 385–397 (1975).
- ⁹ Spiess, F. N. & Mudie, J. D. *The Sea* (ed. Maxwell, A. E.) 205–220 (John Wiley and Sons, New York, 1970).
- ¹⁰ Laird, N. P. *J. Mar. Res.* **29**, 226–234 (1971).
- ¹¹ Detrick, R. S., Williams, D. L., Mudie, J. D. & Sclater, J. G. *Geophys. J.R. astr. Soc.* **38**, 627–637 (1974).
- ¹² Lupton, J. E., Weiss, R. F. & Craig, H. *Nature* **267**, 603–605 (1977).
- ¹³ Klitgord, K. D. & Mudie, J. D. *Geophys. J.R. astr. Soc.* **38**, 563–586 (1974).
- ¹⁴ Lonsdale, P. *Geology* **5**, 147–152 (1977).

in brines associated with salt domes⁶ shows that helium is the only component in the Red Sea brines which unequivocally requires derivation from hydrothermal circulation of seawater in basalts. The helium isotopes are thus an extremely powerful and sensitive tracer for the detection and mapping of hydrothermal systems in oceanic spreading centres.

The Red Sea brines represent an extreme example of the most probable mechanism for the actual injection of mantle helium into the deep sea, namely the penetration and hydrothermal circulation of seawater in basalts on the crests of mid-ocean rises. In the Red Sea the circulating fluid is a brine because the downward penetrating seawater encounters evaporites before reaching the basalt⁶, and thus the upward convecting fluid is stabilised in brine pools in depressions on the sea floor. In normal oceanic spreading centres, however, where evaporites are absent, the heated seawater should emerge as hydrothermal plumes which should be detectable by the association of temperature anomalies with the mantle helium signature. In this letter we report the first He³ measurements on such plume samples, showing that hydrothermal circulation of seawater in basalts is an active process at spreading centres which transfers mantle helium to deep ocean water.

In May 1976 nine samples of bottom water were collected along the central axis of the Galapagos Spreading Center^{7,8} using a newly-devised hydrographic sampling sled^{9,10} attached to the Scripps Institution of Oceanography deep-tow instrument. The location of the samples relative to the local topography is described in the accompanying paper¹⁰. Three of these samples were collected during 'hydrothermal spike' temperature excursions whose origin could not be explained by turbulent mixing within the ambient water mass. The samples were taken about 10 m above the axial fissure opening, at the same level as the CTD sensor which recorded the temperature anomalies. When the sled was recovered, samples for helium analysis were sealed in copper tubing pinch-clamp containers and returned to the laboratory for analysis on a ³He/⁴He ratio mass spectrometer as described previously^{3,5}. Radon activities were measured at sea on six of these samples, using our normal shipboard extraction and counting techniques¹¹ with the exception that the analyses were made on one, rather than twenty, litres of water.

Table 1 lists the ³He results on the three samples taken during temperature spikes (7–0, 8–3, and 8–6) and on the samples of normal or 'background' bottom water where no temperature anomalies were observed on the continuous tow record. The measurements are tabulated as percent deviations of the ³He/⁴He ratio (*R*) from the atmospheric ratio (1.40 × 10⁻⁶), that is

$$\delta(^3\text{He}) = 100 [(R/R_{\text{atm}}) - 1]$$

The mean background water anomaly is $\delta(^3\text{He}) \approx 30.4\%$, in excellent agreement with values of 30.3 and 31.0% observed on the crest and at the mid-depth ³He maximum on the eastern flank of the East Pacific Rise, south and west of the present area². Relative to this background water, which is presumably the water entering the hydrothermal system, the ³He/⁴He ratio in sample 7–0 is enriched by 53% (1.99/1.30). The other two plume samples are also enriched in ³He relative to ambient background, but by much smaller amounts. The $\delta(^3\text{He})$ value of 99% measured on sample 7–0 is by far the largest value yet observed in ocean waters, and establishes for the first time a clear connection between the mantle helium in oceanic basalts and the excess ³He observed in the deep oceans, by seawater penetration and circulation in the basalts.

Radon concentrations (Table 1) are reported as 'excess radon' relative to the activity of ²²⁶Ra. The ²²⁶Ra activity was assumed to be 33 d.p.m. per 100 kg from a previous study by this laboratory¹¹ on bottom waters 29 km south-west of the present location in approximately the same depth; rough measurements made on the present small samples were in

Mantle helium in hydrothermal plumes in the Galapagos Rift

THE ³He/⁴He ratio in deep Pacific water is 20–30% higher than in atmospheric helium because of injection of primordial helium from the mantle^{1,2}. The largest ³He enrichments in the Pacific have been found in water on the crest of the East Pacific Rise where the isotopic ratios indicate³ that the excess helium component has a ³He/⁴He ratio about ten times the atmospheric ratio, in agreement with the ratios measured in trapped helium in the glassy rims of oceanic tholeiites^{3,4}. Recent measurements in this laboratory⁵ have shown that the hot brines in the axial rift of the Red Sea are very highly enriched in mantle helium. ³He and ⁴He are respectively 3300 and 380 times supersaturated relative to atmospheric solubility equilibrium in seawater, with a ³He/⁴He ratio of 1.2 × 10⁻⁶, or 8.6 times the ratio in atmospheric helium. Comparison of the enrichments of various elements in the Red Sea brines and

Table 1 ^3He and ^{222}Rn measurements on hydrothermal plume and background water on the Galapagos Rift ($\sim 0^\circ 48' \text{N}$, $85^\circ 55' \text{W}$)

Tow no., bottle no.	$\delta(^3\text{He})$ (%)	Excess ^{222}Rn (d.p.m. per 100 kg)
<i>a</i> Samples with potential-temperature spikes		
7-0*	99.3	191
8-3	35.7	80
8-6	42.6	229
<i>b</i> 'Background water'		
7-1	27.8	39
7-2	—	49
8-1	28.6	—
8-2	30.2	33
8-5	33.4	—
8-7	32.2	—
Mean	30.4 ± 2	40 ± 8

Radon precision = ± 3 d.p.m. per 100 kg (counting error); ^{226}Ra activity assumed = 33 d.p.m. per 100 kg. $^3\text{He}/^4\text{He}$ ratios are accurate to $\pm 0.7\%$ in $\delta(^3\text{He})$.

*The temperature spike for sample 7-0 is shown in Fig. 1 of ref. 10; samples 8-3 and 8-6 were collected from plumes showing smaller temperature spikes.

agreement within the larger errors. The excess radon concentrations in the three plume samples (80–229 d.p.m. per 100 kg) are clearly much greater than the background value of about forty, although they do not scale with the ^3He anomalies. Because of the short radioactive mean life (5.5 d) the ^{222}Rn enrichments in plume waters are probably due to extraction of radon from the basalts, although some contribution from sediment material trapped within the axial fissure cannot be excluded.

Fig. 1 shows the absolute ^3He and ^4He concentrations in the deep tow samples, together with atmospheric solubility values and vectors for addition of atmospheric helium to air-saturated deep water, and for addition of radiogenic ^4He and pure 'mantle helium' (basalt values^{3,4}) to background water on the Galapagos spreading centre. (The 'absolute' concentrations, based on preliminary 'peak-height' determinations for ^4He , are accurate to about 5%; more accurate isotope dilution measurements are in progress.) ^3He and ^4He enrichments in the ambient background water relative to equilibrium solubility represent the net effect of air injection¹² and addition of mantle

and radiogenic helium to deep water during its circulation. The absolute helium anomalies in sample 7-0 are very much larger: ^3He and ^4He concentrations are respectively 75% and 14% greater in this sample than in the mean Galapagos background water.

The added increment of ^3He relative to background water is $5.9 \times 10^{-14} \text{ cm}^3 \text{ STP per g}$ in sample 7-0, an amount approximately equal to the initial solubility equilibrium (Fig. 1) and thus about 0.03% of the ^3He concentration observed in the Red Sea brines. The estimated temperature of inflowing Red Sea brines is $\sim 100^\circ \text{C}$ (ref. 13); thus if the hydrothermal systems in Red Sea and Galapagos basalts are at all similar, a temperature spike of $\sim 0.03^\circ \text{C}$ is predicted for sample 7-0, in reasonable agreement with the signal observed during sampling¹⁰.

In Fig. 1 the vector from the mean background water to sample 7-0 corresponds to addition of helium with $^3\text{He}/^4\text{He} = 9.4 \times 10^{-6}$ or $R = 6.7 R_{\text{Atm}}$, significantly lower than the 'mantle helium' ratio ($R \sim 10 R_{\text{Atm}}$) observed in ocean-ridge basalts^{3,4}, which implies that about 30% of the added helium is radiogenic ^4He generated in the oceanic crust. Helium with $R = 6.7 R_{\text{Atm}}$ would be found in Pacific ridge-basalts after about 30 my of ^4He production (ref. 4), an age far too great for basalts in such close proximity to the rift axis. The holocrystalline portions of pillow basalts exchange trapped gases with seawater on a much shorter time scale than this¹⁴; thus the helium isotope ratios in basalt-seawater hydrothermal systems probably decrease continuously with time as the initial mantle helium component is progressively stripped out by the fluid phase and the radiogenic component grows in. It may therefore be possible to characterise such hydrothermal systems in time and space by detailed mapping of helium isotope ratios in plume samples.

The extreme sensitivity of helium and radon for plume detection is indicated by our estimate that sample 7-0 contained of the order of 0.03% of hydrothermal water. A sample with a 1°C temperature spike would, on this basis, contain helium with $R \sim 5.5 R_{\text{Atm}}$ and a ^{222}Rn activity of $\sim 5,000$ d.p.m. per 100 kg. Radon is of course a radiogenic isotope which is present in very high activity in marine sediments; it is therefore not a completely unambiguous tracer for seawater which has penetrated basalts. The ^3He signature, however, provided unequivocal evidence for the existence of a hydrothermal basalt-seawater system in the axial region of the Galapagos Rift.

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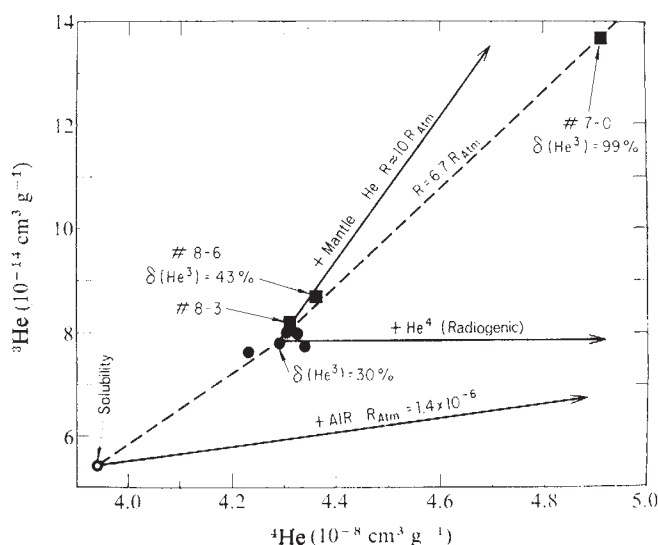
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- 1 Clarke, W. B., Beg, M. A. & Craig, H. *Earth planet. Sci. Lett.* 6, 213 (1969).
- 2 Craig, H., Clarke, W. B. & Beg, M. A. *Earth planet. Sci. Lett.* 26, 125 (1975).
- 3 Lupton, J. E. & Craig, H. *Earth planet. Sci. Lett.* 26, 133 (1975).
- 4 Craig, H. & Lupton, J. E. *Earth planet. Sci. Lett.* 31, 369 (1976).
- 5 Lupton, J. E., Weiss, R. F. & Craig, H. *Nature* 266, 244 (1977).
- 6 Craig, H., *Hot Brines and Recent Heavy Metal Deposits in the Red Sea*, (eds Degens, E. T. and Ross, D. A.) 208–242 (Springer-Verlag, New York, 1969).
- 7 Sclater, J. G. & Klitgord, K. D. *J. geophys. Res.* 78, 6951 (1973).
- 8 Williams, D. L., Von Herzen, R. P., Sclater, J. G. & Anderson, R. N. *Geophys. J. R. Astr. Soc.* 38, 587 (1974).
- 9 Weiss, R. F. *et al.*, *Trans. Am. Geophys. Union* 57, 935 (1976).
- 10 Weiss, R. F. *et al.* *Nature* 267, 600–603 (1977).
- 11 Chung, Y.-C. & Craig, H. *Earth planet. Sci. Lett.* 14, 55 (1972).
- 12 Craig, H. & Weiss, R. F. *Earth planet. Sci. Lett.* 10, 289 (1971).
- 13 Ross, D. A. *Science* 175, 1455 (1972).
- 14 Dymond, J. & Hogan, L. *Earth planet. Sci. Lett.* 20, 131 (1973).

Fig. 1 ^3He and ^4He concentrations in water samples with temperature spikes (squares) and in ambient 'background water' (circles) on the axial fissure of the Galapagos Rift. The point labelled 'solubility' indicates the equilibrium air saturation concentrations for deep water. ● Normal bottom waters; ■ Hydrothermal plumes.



High explosive analogue of the Tunguska event

THE Tunguska event of 1908, which has been described in detail by Krinov¹, is generally accepted as having been caused by a detonating bolide. Zotkin and Tsikulin² concluded, on the basis of laboratory experimentation, that the event could best be fitted by a travelling wave striking the forest at an angle of 30° to the horizon, culminating in a detonation at a height roughly eight times that of the forest canopy. The pattern of tree blowdown consisted of a radially symmetric zone 35 km in diameter, on which was superimposed a bi-lobed pattern caused by the travelling wave. Stanyukovich and Bronshten³ quote a total energy of 10^{23} erg for the event. In August 1966 an experiment was carried out in a mixed forest of Pine, Spruce and Fir near Hinton, Alberta, Canada, which has some features in common with the Tunguska event. In the experiment a hemispherical stack of TNT, 100,000 lb (50 short tons) in weight, was detonated on the forest floor. The resulting pattern of tree blowdown is shown in Fig. 1.



Fig. 1 Effect of 50-t charge in a mixed forest.

The blowdown zone is 130 m in diameter, and may be compared with the zone of symmetric blowdown at Tunguska. Simple cube root scaling of the dimensions lead to an estimate of 10^5 tons TNT for the Tunguska event. On the basis of a 50% partition of energy, this is equivalent to a 200-kiloton (kt) nuclear event. Taking 1.2 kcal g^{-1} for TNT, the estimated energy for Tunguska is 4×10^{21} erg, two orders of magnitude smaller than the estimate given above. This lower estimate, however, may be a close approximation to the energy of the final detonation, that is to say ignoring the contribution from the travelling wave.

Note, however, that the experiment was a surface explosion, whereas the Tunguska event is believed to be a low 'air burst'. Thus, a refinement of the estimate could be made using the standard height of burst curves. The expected difference in the pattern of blowdown at the full scale—that is, due to a 200-kt detonation at, say, 200 m height—is unlikely, however, to approach an order of magnitude, and will probably be closer to unity. In view of the uncertainties inherent in comparing conventional, nuclear and kinetic energy events in terms of their residual effects, such a refinement of the calculation would be quite unjustified.

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¹ Krinov, E. L. *Giant Meteorites* (Pergamon, Oxford, 1966).

² Zotkin, I. T. & Tsikulin, M. A. *Dok. Akad. Nauk S.S.S.R.*, **167**, 59–62 (1966).

³ Stanyukovich, K. P. & Bronshten, V. A. *Dok. Akad. Nauk S.S.S.R.*, **140**, 583–586 (1961).

Body-centred cubic cylinder packing and the garnet structure

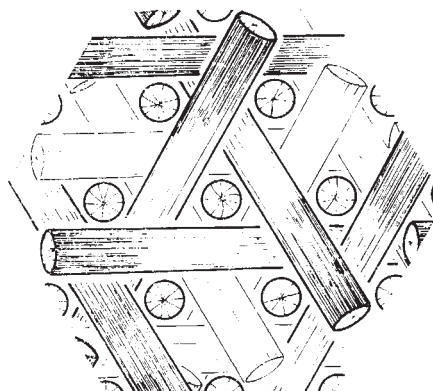
ANALYSIS of the symmetry of periodic packings of identical (circular) cylinders has been found to be useful in analysis of crystal structures. To provide a description of a crystal structure, the cylinders are replaced by rods¹ of atoms or groups of atoms. The densest cubic packing of cylinders is of particular interest: we show how the garnet structure can be described simply in terms of this packing.

Periodic packings of simple geometric objects such as spheres and polyhedra have long had an important role in crystal structure description but little attention has been paid to the problem of cylinder packings in this connection. Periodic packings of cylinders with parallel axes or with axes in parallel planes are fairly easy to visualise, but packings with cubic symmetry are less obvious. One of these, described here, is of special importance to the description of complex cubic structures previously considered enigmatic.

In this packing, cylinders in contact with each other lie with their axes parallel to four cubic $\langle 111 \rangle$ directions. A sketch of a fragment of the packing is shown in the figure. The space symmetry group² of the arrangement is $I4_1/a\bar{3}2/d$ ($Ia3d$) so we propose to call it body-centred cubic cylinder (or rod) packing. The fraction of space filled by cylinders in contact is $\sqrt{3}\pi/8 = 0.680$; this is considerably less than for the densest cylinder packing (which is the familiar honeycomb arrangement with hexagonal symmetry) for which the space-filling fraction is $\pi/\sqrt{12} = 0.907$. Nevertheless it seems to be a safe conjecture that this is the densest cubic packing of identical cylinders.

In applying the cylinder packing to crystal structure description we notice that each cylinder axis lies on a threefold symmetry axis so that a characteristic feature of the symmetry is the presence of four non-intersecting threefold axes. Crystal structures that will lend themselves to description in terms of

Fig. 1 A fragment of body-centred cubic cylinder packing viewed down a trigonal axis.



body-centred cubic rod packing will therefore have this symmetry feature: their space group will be one of the cubic subgroups of $Ia3d$. These are $I\bar{4}3d$, $I4_132$, $Ia3$, $I2_13$, $Pa3$, $P4_132$, $P4_332$, and $P2_13$.

A good example of a crystal structure that can be described as a body-centred cubic rod packing is that of the garnets (symmetry $Ia3d$) typified by grossularite, $\text{Ca}_3\text{Al}_2\text{Si}_3\text{O}_{12}$. The structure³ has been known for fifty years, but has so far resisted a simple description. In this structure the anions are arranged around the threefold axes to form infinite rods of alternating and nearly regular triangular prisms (empty) and octahedra (centred by aluminium ions). Arranging such rods in body-centred cubic rod packing accounts for all the anions in the structure and leaves available sites of eightfold and tetrahedral coordination to accommodate the calcium and silicon ions respectively. The cation positions are fixed by the high symmetry so that an 'ideal' garnet structure can be derived in which the rods consist of alternating regular octahedra and prisms of anions. The coordinates of the 96 anions in the unit cell in the general symmetry-related positions $96h$ are then

$$x = (2\sqrt{3} - 3\sqrt{2} + \sqrt{6} - 2)/8 = -0.0411$$

$$y = (\sqrt{6} - 2)/8 = 0.0562$$

$$z = (3\sqrt{2} - 2\sqrt{3} + \sqrt{6} - 2)/8 = 0.1535$$

very close to those found for real crystals, e.g., for berzeliite⁴ ($x = -0.0391$, $y = 0.0522$, $z = 0.1568$).

Other cubic crystal structures that may be described similarly as a cubic rod packing include those of Th_3P_4 (space group $I\bar{4}3d$), $\beta\text{-Mn}$ ($P4_132$), RbAg_4I_5 ($P4_132$), and bixbyite ($Ia3$). A full description of these and related structures and of rod packing in general is in preparation. It would be of interest also to explore the possibility of cubic rod packings occurring in assemblies of rod-shaped biological molecules or even in the structure of organic matter at a high level such as cellular substructure.

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¹ Shubnikov, A. V. & Kopstik, V. A. *Symmetry in Science and Art* ch. 6 (Plenum, New York, 1974).

² *International Tables for X-ray Crystallography* 1 (Kynoch, Birmingham, 1965).

³ Menzer, G. Z. *Kristallogr.* 63, 157 (1926); 69, 300 (1928).

⁴ Hawthorne, F. C. *Acta Cryst.* B32, 1581 (1976).

Behaviour of mercury species in isolated estuarine sediment samples—growth and decay of methyl mercury during storage

WE have investigated the levels of methyl mercury found in mercury-containing sediments from the River Mersey Estuary, England, as part of our work on the determination and transport of mercury in the environment¹. We now wish to draw attention to the unexpected behaviour of sediment-bound methyl mercury on storage. In the initial stages of this study we experienced difficulty in obtaining consistent methyl mercury values; supposedly identical samples analysed at intervals of a few days gave markedly different results. We, therefore, followed the levels of methyl mercury in selected sediments over a period, to

determine if any change was occurring on storage. We found that the amounts of methyl mercury observed in the stored sediments did not remain constant; initially there is a rise in the amount of methyl mercury observed, and then, after about ten days, the amount present begins to decline to levels which in general only approximate to those originally present. We have observed this phenomenon in nearly all of the Mersey sediments samples we have examined; in two cases where the total mercury levels were $< 1.5 \mu\text{g g}^{-1}$, a rise in methyl mercury levels was not detected. In general the sediments were in fact found to contain quite high total mercury levels (usually between 5 and $8 \mu\text{g g}^{-1}$) and this feature may be a requirement for the effect to be observed.

For each experiment, a bulk sample of sediment was taken from the intertidal zone of the River Mersey Estuary. This sample (of several kilograms weight) was immediately homogenised (by vigorous hand mixing) on site, and divided into a number of subsamples, each of about 120 g. Two or three of these were immediately frozen with solid carbon dioxide or liquid nitrogen.

On return to the laboratory, the frozen samples were thawed and analysed for methyl mercury. The values obtained were considered to be the ambient levels present in the sediment in estuarine conditions. The other samples were analysed at regular intervals, and the methyl mercury found was plotted against the time elapsed since they were collected. The samples were stored in the laboratory, in darkness, and samples were also analysed for total mercury as previously described¹. The samples were sealed in glass containers in anaerobic conditions at room temperature (18°C). In this, and in nearly all other cases, we observed that on analysis over a period of about ten days, the initially identical samples in fact revealed quite different methyl mercury contents. During storage over this period the level of methyl mercury increased quite significantly until a maximum level was observed after the ten-day period. This maximum is normally about 2–4 times that present initially, although on occasions increases between 10 and 15 times have occurred. After about 10 days the level of methyl mercury declined to an equilibrium level over a period of about 30–40 days. Samples were taken from three different locations in the estuary; Hale Point (Map Ref. SJ 471808), Fiddlers Ferry (SJ 563865) and Stanlow Marshes (SJ 448788), and showed similar results. The total mercury level was usually between 5 and $8 \mu\text{g g}^{-1}$. Samples were obtained on four separate occasions during 1975 and 1976.

The method used for the analysis of methyl mercury was adapted from that of Uthe, Solomon and Grift². Sediment samples of 2–5 g were extracted with toluene after treatment with copper sulphate and an acidic solution of potassium bromide. Methyl mercury was then back extracted into aqueous sodium thiosulphate. This was then treated with acidic potassium bromide and copper sulphate following which the methyl mercury was extracted into pesticide grade benzene containing approximately $100 \mu\text{g dm}^{-3}$ of ethyl mercuric chloride as an internal standard. The extract was analysed by electron capture gas chromatography using a Pye 104 chromatograph equipped with a nickel 65 detector. The glass column ($1\text{ m} \times 0.4\text{ cm}$) was packed with 5% neopentyl glycol adipate on Chromosorb G (AW-DMCS). Methyl mercury was measured by comparing the peak heights with standards of methyl mercuric chloride made up in the ethyl mercury–benzene solution. The results were calculated as nanograms of methyl mercury per gram of dry sediment. The detection limit was $1\text{--}2\text{ ng g}^{-1}$. Six replicate analyses of a sample gave a mean value of 15.78 ng g^{-1} with range limits of $15.39\text{--}16.34\text{ ng g}^{-1}$. Recovery of 20 ng of methyl mercuric chloride added in aqueous solution to the sediment at the start of the procedure was better than 97%.

It should be pointed out that no mercury species has been added to these samples during or after collection. Therefore the observation of a growth and decay curve similar in both magnitude and time scale to those observed by other workers³⁻⁶, who had incubated sediments with separately added inorganic mercury compounds, was unexpected. There has been no previous report of growth of methyl mercury in stored sediments unless inorganic mercury has been added during the experiment, and these observations are therefore of relevance to analytical work on samples containing methyl and inorganic mercury. It has also been noted that sediments sterilised, normally by autoclaving at approximately 120 °C, did not produce methyl mercury on incubation with inorganic mercury³. This suggested a microbiological origin for the methyl mercury produced from the previous samples.

A control experiment was then carried out in which a large number of identical samples were collected and homogenised. Half of the samples were sterilised by treatment with an approximate 4% w/w solution of formaldehyde. We did not wish to carry out the sterilisation by autoclaving as this would have led to loss of the methyl mercury initially present and also to a substantial reduction in the amount of total mercury present. Several samples of both sterilised and unsterilised sediments were analysed each day. (This duplication was to compensate for any variation between individual samples.) All of the samples were stored at ambient room temperature (18 °C) in the laboratory. It can be seen from Fig. 1 that there is a

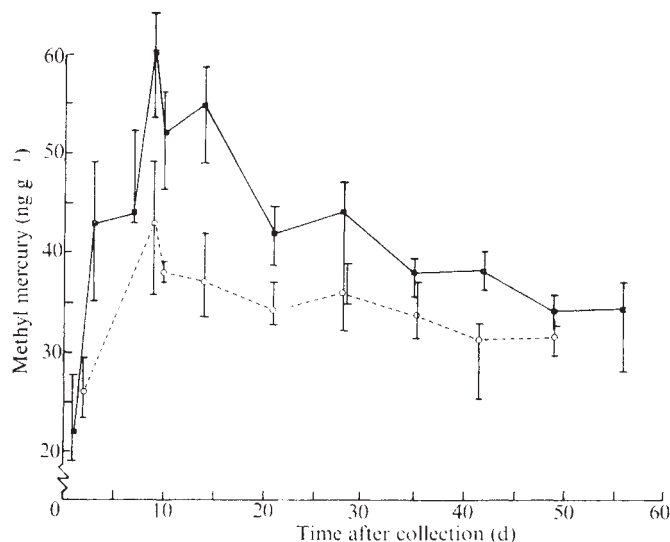


Fig. 1 Analyses of sterilised and unsterilised sediments from Hale Point, for methyl mercury. Total mercury is $7.24 \mu\text{g g}^{-1}$. Results up to day 25 are the mean of eight determinations; results beyond day 25 are the mean of four determinations. Error bars represent range limits for each analysis series. The samples were stored at room temperature (18 °C). —■—, Un-treated; ---○---, sterilised samples.

difference in behaviour between the sterilised and unsterilised samples. In addition some of the samples were separately inoculated into various growth media to test for microbiological activity. With the unsterilised samples prolific growth occurred in both aerobic and anaerobic media, whereas the sterilised sediment samples showed no growth in either type of medium. This confirms that the biological activity in the control samples had been destroyed by the sterilisation process used, and Fig. 1 indicates that the initial levels of methyl mercury were not significantly affected.

The growth and decay curve observed in the Fig. 1 for the unsterilised samples was of the same character as we have observed above. But the sterilised samples behaved in an unexpected way in also showing a growth and decay curve, although the maximum level of methyl mercury achieved was significantly smaller.

These observations may be best understood by a consideration of the process of methyl mercury generation in sediments. An equilibrium probably exists between production of methyl mercury from the large excess of inorganic mercury present in the sediments and loss of methyl mercury by evaporation, solution into sediment pore water or by bacteriological degradation⁴⁻⁷. It has also been stated that tidal or other disturbance of natural sediments increases their methylating rates⁸. Such a disturbance would occur on collection and correlates well with our observed initial increase in methyl mercury. When a certain level is reached, however, the methyl mercury then apparently acts as a poison to the methylating species⁹. The mechanism for loss of methyl mercury, however, would continue to operate, possibly in modified form, causing the level to fall gradually. Finally a new equilibrium seems to be reached, but this new steady-state level does not seem to correspond with that found in the estuary in normal conditions.

To explain the smaller growth of methyl mercury in the sterilised samples we suggest that the methylating species (e.g. methyl corrinoids¹⁰) existing in the sediments persists even after the associated organisms that produce it are destroyed by the sterilisation process. This residue then reacts with the reservoir of inorganic mercury present to produce the initial rise again, until it is exhausted and a loss mechanism for methyl mercury predominates. Whereas for the unsterilised samples the methylating species may be biologically generated to allow methylation to continue, this cannot occur for sterilised samples. This scheme fits well the suggested mechanism of methylation as a chemical process with an enzymatic generation of the methylating species¹¹. It should be noted that the loss mechanism for the sterilised samples cannot involve bacteriological activity and this may account for the slightly slower decay rate of methyl mercury in this case. It should also be pointed out that as sterilisation by autoclaving will also destroy the majority of the enzymes and co-enzymes present, no methylation after this method of sterilisation would be expected.

The appearance of excess methyl mercury on incubation of a natural mercury-containing sediment in the laboratory is significant to the previous work on mercury methylation. It suggests that the application of laboratory-derived results directly to natural conditions could, in these cases, be misleading; our analytical results for day 10 if extrapolated directly might lead to the conclusion that natural methyl mercury levels and rates of methylation are much greater than in fact they really are. Work in this area with model or laboratory systems needs to be interpreted with particular caution. As our observations follow the same pattern for samples of different bulk, stored in different containers and at different temperatures, this also suggests that for properly meaningful survey work for methyl mercury the analytical work must be performed within a short time of collection. In the cases where we have not observed the normal pattern, the total mercury concentrations were relatively low. It is possible therefore that these phenomena may only be observable when high concentrations of mercury and organic material are present, that is, in conditions of severe mercury contamination of an already polluted waterway.

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- ¹ Craig, P. J. & Morton, S. F. *Nature* **261**, 125–126 (1976).
- ² Uthe, J. F., Solomon, J. & Grift, B. J. *Assoc. Offic. Anal. Chem.* **55**, 583–589 (1972).
- ³ Jensen, S. & Jernelov, A. *Nature* **223**, 753–754 (1969).
- ⁴ Spangler, W. J., Spigarelli, J. L., Rose, J. M. & Miller, H. M. *Science* **180**, 192–193 (1973).
- ⁵ Olsen, B. H. & Cooper, R. C. *Nature* **252**, 682–683 (1974).
- ⁶ Olsen, B. H. & Cooper, R. C. *Water Research* **10**, 113–116 (1976).
- ⁷ Wood, J. M. *Rev. Intern. Oceanogr. Med. Tomes XXXI–XXXII*, 7–16 (1973).
- ⁸ McEntyre, F. E. & Neufeld, R. D. *Water Pollut. Control* **74**, 465–470 (1975).
- ⁹ Billen, G., Joiris, C. & Wollast, R. *Water Research* **8**, 219–225 (1974).
- ¹⁰ Imura, W., Pan, S.-K., Shimizu, M. & Ukita, T. *New Methods Environ. Chem. Toxicol. Collect. Paper Res. Conf. New Methods Ecol. Chem.* (ed. Coulston, F.) 211–216 (Int. Acad. Print Co. Ltd., Tokyo, Japan, 1973).
- ¹¹ Wood, J. M., Kennedy, F. S. & Rosen, C. G. *Nature* **220**, 173–174 (1968).

Maya Formative phase radiocarbon dates from Belize

THE Formative phase of Maya civilisation in Central America, until recently thought to date back no further than ~ 900 BC, has been extended to at least 2,500 BC by a series of radiocarbon dates from the site of Cuello, Belize, on samples from the 1975 excavations¹. The series included two dates of 1,020–1,050 b.c. (1,250–1,300 BC) from upper levels at the site and one date of 2,050 b.c. (2,600 BC) from a midden overlying the buried land surface. (The convention b.c. for radiocarbon years and BC for calendar years using the bristlecone pine calibration curve is followed here; for the calibration schemes used, see refs 2 and 3.) We report here a further series of 16 dates on samples from the 1976 excavations at Cuello, which confirm the early date for the Maya Formative and which also suggest occupation of the site at an even earlier date, ~ 3,200 b.c. (4,000 BC).

The florescence of Classic Maya civilisation in the first millennium AD, in the arid Yucatan Peninsula of Mexico and the tropical rainforest of Guatemala and Belize to the south, was preceded by a Preclassic or Formative period still of unknown persistence, beginning with the initial settlement of the Maya lands by sedentary farmers. The Formative is defined by the presence of an agricultural economy, based in Mesoamerica in general on maize, beans, avocado, peppers, squash and root crops. The history of plant domestication in Mesoamerica has been taken back to the eighth millennium b.c. by work in Oaxaca⁴ although settled village life is not known before the third millennium b.c. The introduction of pottery, which is a good indicator of settled life because of its fragility and relative non-transportability, is dated between 2,500 and 2,000 b.c. (3,250–2,500 BC) in several regions of Mesoamerica. The emergence of the first recognisably complex society, that of the Olmec, marked by monumental sculpture and public architecture, occurred after 1,200 b.c. (1,500 BC)⁵.

The site of Cuello, located in northern Belize (formerly British Honduras) at approximately 18°05'N, 88°35'W, is a small Classic Maya ceremonial precinct and settlement located on the crest of the low limestone ridge between the parallel courses of the Rio Hondo and the Rio Nuevo, which flow east of north into Chetumal Bay on the Caribbean coast of the Yucatan Peninsula. To the south of the Classic site, so far only cursorily investigated, lies a series of large platforms; one of these, designated Platform 34 on the site map made in 1975–76 by M. Walton, B. Ah and F. Johnson (Fig. 2 in ref. 6), was investigated in 1975 under the supervision of D. Pring, and yielded the first stratified Early Formative cultural sequence in the Maya Lowlands. A second season of excavation in 1976, supervised by S.D. with the assistance of N. Fradgley and G. Kelsey, resulted

in the exposure of a sequence of buildings set around open patios which are, on the basis of the radiocarbon chronology reported here, the earliest known in the Maya area and among the earliest in Mesoamerica.

The samples for dating were collected during excavation from structural, building fill and midden contexts. In general the concordance between stratigraphy and chronology indicates that organic material redeposited in fill was so used within a short time of the original deposition, but in the case of the three earliest dates reported here, which are stratigraphically anomalous, redeposition many centuries later is posited.

The dates obtained on the 1976 excavation samples are shown in Table 1 together with an indication of the context, associated ceramics and major cultural period, and calibration of the dates on the schemes of Ralph *et al.*² and Clark³. They are also shown in Fig. 1 with the range of 1 and 2 s.d. indicated, correlated with the phases of building construction I–VIII elucidated in the excavation, which form a stratigraphic control on the radiocarbon chronology.

Radiocarbon determinations were made in Los Angeles by R.B. and S. de A. and in Cambridge by V.R.S. and A.P.W., using gas proportional detectors filled with purified carbon dioxide produced by the oxidation of the specimens of partially burnt wood from the excavations. Traces of structural plaster were eliminated by treatment

Fig. 1 Radiocarbon chronology for the Formative Period sequence at Cuello, Belize, ranged against a calibrated scale in calendar years (at right, after Clark³) and construction phases O–IX elucidated in excavation. O, old land surface; D, destruction level. The period of erection of the first dated Maya monuments AD 260–300 is also indicated. Laboratory references and uncertainties are shown in Table 1.

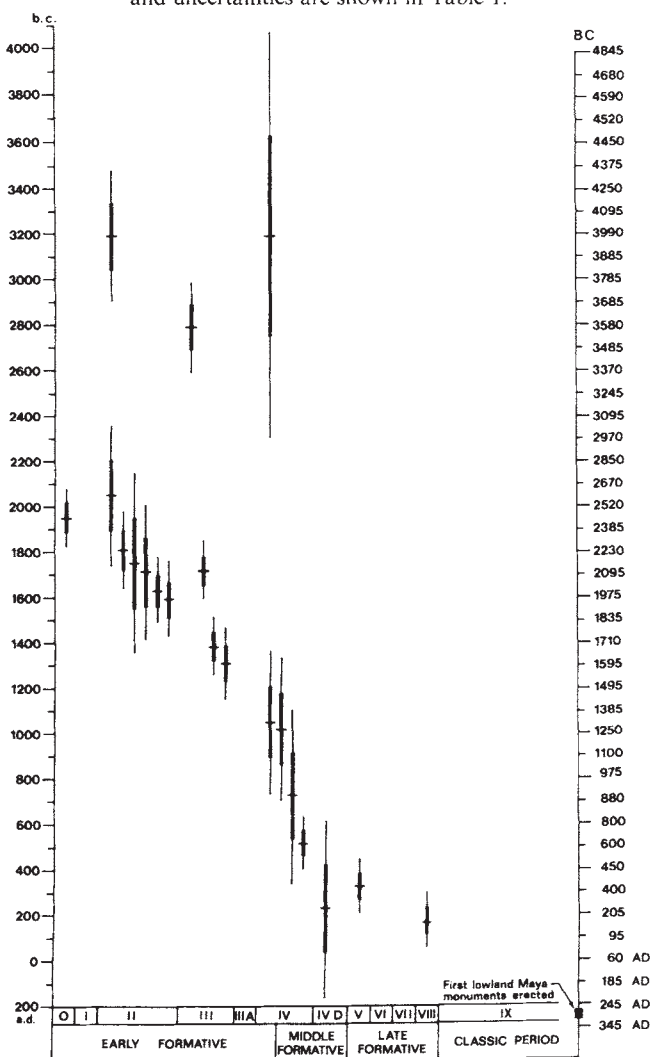


Table 1 Radiocarbon dates from Cuello, Belize, in numerical order of excavation provenance, with context, associated ceramics where present, and major cultural period indicated

Provenance (Op. 17F levels)	Context	Ceramic complex	Major period Maya Valley of area Mexico	Laboratory reference	Conventional ¹⁴ C date b.p. b.c. ±	MASCA 1σ BP	Clark 1σ BP	Clark 1σ BC	Clark 2σ BP	Clark 2σ BC	Archaeological assessment
34	F	Co	Middle/Late Formative Early hori- zon and First Intermedi- ate Period	UCLA-2102f	2280 330 60	2395-2350	2410-2320	460-370	2520-2130	570-180	Acceptable
76	F	Lo		UCLA-2102a	2470 520 60	2740-2450	2750-2430	800-480	2810-2380	860-430	Acceptable
110	S	Lo		UCLA-2102g	5140 3190 445	6420-5450	6430-5470	4480-3520	(6930)-4910	(4980)-2960	Redeposited??
141	F	Lo		UCLA-2102h	2190 240 195	2435-1940	2430-1870	AD 480-80	2780-1725	AD 830-225	Acceptable
143	S	—		UCLA-2102e	2690 740 70	2960-2120	2950-2790	1000-840	3060-2720	1111-770	Contamination?†
155	S	Sw		UCLA-2102b	4740 2790 100	5630-5340	5640-5335	3700-3385	5775-5250	3825-3300	Redeposited??
175	F	Sw		Q-1579	3260 1310 80	3660-3460	3685-3445	1735-1495	3830-3320	1880-1370	Acceptable
176	S	—		Q-1574	3670 1720 65	4120-4060	4230-3935	2280-1985	4395-3780	2445-1830	Acceptable
189	D	Sw		Q-1573	3580 1630 70	4090-3990	4415-3840	2465-1890	4735-3580	2785-1630	Acceptable
189	D	Sw		UCLA-2102d	3700 1750 200	4490-3950	4100-3805	2150-1855	4260-3665	2310-1715	Acceptable
197	S	Sw	Early Formative	Q-1578	3340 1390 65	3820-3590	3785-3520	1835-1570	3940-3400	1990-1450	Acceptable
210	S	Sw		UCLA-2102c	5140 3190 145	6140-5710	6105-5770	4155-3830	6330-5610	4380-3660	Redeposited??
211	S	Sw		Q-1577	3550 1600 85	4060-3870	4055-3765	2105-1815	4210-3625	2260-1670	Acceptable
219	S	Sw		Q-1576	3660 1710 150	4250-3990	4290-3850	2340-1900	4750-3645	2620-1695	Acceptable
230	O/S	Sw		Q-1571	3900 1950 65	4530-4160	4555-4245	2605-2295	4745-4070	2795-2120	Acceptable
239	M	Sw		Q-1572	3760 1810 85	4390-4100	4360-4035	2410-2085	4515-3960	2565-2010	Acceptable

Contexts F, fill; S, structural; D, destruction; O/S, occupation/old soil; M, midden. Ceramic complexes—Co, Cocos; Lo, Lopez; Sw, Swasey. MASCA refers to Ralph et al.²

* Datings considered too early for archaeological context.

† Accidental contamination suspected.

with dilute mineral acid, and any humic acid was removed with dilute alkali. Some samples were rather small, and where they yielded insufficient gas to fill the counters, radioactively inert carbon dioxide was added. Samples were counted to a statistical accuracy of 1 s.d. (1σ). The calculated radiocarbon dates quoted are based on the 'Libby' half life for radiocarbon of 5,568 ± 30 yr. Measurement of the stable isotopes ratio ¹³C/¹²C was made on the gas produced from several of the dated samples. The results were very consistent and indicate that samples were of charcoal from normal wood and were not contaminated by carbon of mineral origin.

Our dates consist of nine from the 1975 excavation¹ and 16 from the 1976 excavation. Of these 25, 18 dates are considered to be archaeologically acceptable in their relationship to the site stratigraphy and to each other. Three dates from the 1975 series (UCLA 1985 bc, d and f) are considered too late by more than a millennium to be acceptable, although no certain cause or source of contamination has been found. One 1976 date (UCLA 2102 e) came from a context later suspected of accidental contamination during excavation, and has been set aside as unreliable. Three other dates from the 1976 series (UCLA 2102 b, c and g) are considered too early for the archaeological contexts from which they were recovered, by more than a millennium; these are tentatively ascribed to redeposition of older material, and are discussed below.

The remaining 18 dates provide an internally and stratigraphically consistent radiocarbon chronology for the Cuello site, and through the associated ceramics for the Formative period in the Maya lowlands. Of the 18 dates, 12 are associated with structures and ceramics of the Early Formative Swasey phase, four with the Middle Formative Lopez Mamom phase and two with the Late Formative Cocos Chicanel phase. On the basis of these dates we can propose chronological limits for the three major divisions of the Maya Formative, and also integrate them into the revised stadial model adopted for the sequence in the Valley of Mexico by some scholars⁷.

The central dates for the 12 determinations attributed to the Early Formative range from 2,050 to 1,050 b.c.; the earliest and latest dates in the series have a 1σ of 155 and 160 yr respectively, and taking 2σ as a reasonable outer limit the Early Formative would fall within the period 2,370-730 b.c. The dates for the Middle Formative suggest that the boundary between the two periods does not fall as late as 730 b.c., and we propose that a boundary close to the latest central date be set, at 1,000 b.c. Similarly, at the lower end of the Early Formative we suggest a consonant boundary of 2,000 b.c., although a lowering of this towards 3,000 b.c. with further investigation would not be unexpected in view of the developed ceramic technology at Cuello by 2,000 b.c. and the possibility of earlier occupation mooted below. In terms of calendar years the Early Formative

would span the period 2,500-1,300 bc, coinciding with the limits proposed for the Initial Ceramic period of Mesoamerica in the Valley of Mexico model.

Two other recent radiocarbon dates from Belize also fall within the Early Formative as defined here: a sample obtained by us from the lowest cultural level of the Barton Ramie site in central Belize, associated with ceramics of the early facet of the Jenney Creek ceramic complex, gave a date 3,200 ± 205 b.p., 1,250 b.c., calibrating on 1σ to 1,790-1,320 bc (Q-1575). Another sample, from a wooden post found in the bank of a canal bordering a man-made raised field complex on the Rio Hondo, a short distance west of the Cuello site, yielded a date of 3,060 ± 230 b.p., 1,110 b.c., calibrating on 1σ to 1,650-1,070 bc (I-7877A) (unpublished results of D. Puleston). The Barton Ramie date indicates that by the end of the Early Formative parts of the eastern Maya lowlands were occupied by people making ceramics in a tradition distinct from that of the Swasey complex, while the date from the Rio Hondo raised field suggests that again by the end of the Early Formative at the latest this intensive and organised method of agriculture was being practised in the lowlands. Mollusc evidence from Cuello indicates that swidden agriculture on the limestone ridge was not practised until the beginning of the Middle Formative (unpublished results of L. H. Feldman) so that intensive cultivation may have preceded extensive methods in this region.

The four dates for the Middle Formative are of 1,020, 730, 520 and 235 b.c., the first of these occurring stratigraphically immediately above the final Early Formative levels at the Cuello site and being associated with a burial accompanied by three pottery vessels of transitional Early-Middle Formative type. The 1σ error of 160 yr on this date matches that on the latest Early Formative determination, and the two dates are statistically inseparable. We feel that the phase boundary of 1,000 b.c. is therefore, within the margin of error, acceptable. The date of 235 b.c. does not necessarily indicate an upper limit for the Middle Formative, since the standard deviation of 195 yr is rather large, but a boundary before this date and the earliest Late Formative date of 330 b.c. may be proposed: we suggest that a limit at 400 b.c. will probably lie within the margin of error of a more accurate boundary, when this is established by further determinations. The Middle Formative would then occupy the period 1,300-450 bc in calendar years.

The two dates associated with Late Formative occupation are of 330 and 175 b.c., the latter again not setting a firm upper limit for the period. This upper limit has conventionally been equated with the first appearance of stone monuments bearing hieroglyphic inscriptions in the Maya script, one of the cultural innovations taken as marking the beginning of the Classic period of Maya civilisation. This boundary is currently set between AD 250 and 300 in

the Maya lowlands, and we therefore suggest that the formal bounds of the Late Formative be set at 400 b.c. (450 bc) to AD 250, the latter limit being calculated only in calendar, and not radiocarbon, years from the basis of the evidence by which it has been set.

Although the Middle-Late Formative boundary and the span of the Late Formative might seem to be based on rather less evidence than for the earliest part of the sequence, the limits which we propose are supported by the radiocarbon chronology for the site of Tikal, Guatemala (19 dates in the Middle and Late Formative) and by smaller numbers of dates from other sites including Dzibilchaltun, Aguacatal, Altar de Sacrificios and San Felipe.

We have therefore suggested that, in comparison with the currently accepted framework for the Formative, the Late Formative should begin some 150 yr earlier, the transition between Middle and Early Formative be placed some 500 yr earlier and the formal beginning of the Early Formative be moved back 1,000 yr, in calendar years.

This latter boundary remains the least certain, however. Three of the radiocarbon dates from the 1976 excavations at Cuello are earlier, at 2,790, 3,190 and 3,190 b.c. (3,575, 3,980 bc). The samples of burnt wood from which the dates were obtained occurred in contexts, in two cases structural and in one building fill, from which both later radiocarbon dates and pottery were obtained that fit well into the proposed Early Formative chronology of the site beginning in 2,000 b.c. (2,500 bc). There is no reason for supposing that the early dates are the result of mineral carbon or laboratory error, and we must thus accept that the burnt wood was indeed of the age indicated by the radiocarbon dates. From this it follows, although there is no external corroborative evidence, that the wood must have been reused or redeposited by the Early Formative inhabitants of the site many centuries after it ceased to grow, and in view of the perishability of uncarbonised wood in a tropical lowland soil, probably many centuries after it was charred. If we accept this, there are two methods by which this charring could have occurred: a natural bush fire, and burning by man, on at least two occasions separated by some four centuries. Although there is no internal evidence to support the anthropogenic origin of the burning rather than the natural, such an explanation would correlate with suggested human interference with the vegetation of Peten, Guatemala, 150 km south-west of the Cuello site (unpublished results of F. Wiseman). In addition, the developed nature of the Swasey ceramic complex at Cuello by 2,000 b.c., and the lack of a credible immediate ancestry elsewhere in the Americas (unpublished results of D. Pring) suggest that earlier occupation of the Maya lowlands, if not the Cuello site itself, remains to be discovered. Although the evidence for human presence in the Maya Lowlands in the fourth millennium bc is still sparse and questionable, it is no more so than the totality of evidence for the Early Formative was only two years ago, and the latter is, we would maintain, now tolerably well established.

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- ¹ Hammond, N., Pring, D., Berger, R., Switsur, V. R. & Ward, A. P. *Nature* **260**, 579–581 (1976).
- ² Ralph, E. K., Michael, H. N. & Han, M. *MASCA Newslett.* 9 No. 1 (1973).
- ³ Clark, R. M. *Antiquity* **49**, 251–266 (1975).
- ⁴ Hammond, N. *Phil. Trans. R. Soc., Lond.* **B275**, 120–128 (1976).
- ⁵ Stuiver, M. *Radiocarbon* **11**, 618–624 (1969).
- ⁶ Hammond, N. (ed.) *Archaeology in northern Belize: Corozal Project 1976 Interim Report* (Centre of Latin American Studies, Cambridge University, 1976).
- ⁷ Wolf, E. R. in *The Valley of Mexico* (ed. by Wolf, E. R.), 16–17 (University of New Mexico Press, Albuquerque, 1976).

Natural selection for energetic efficiency and the relationship between activity level and mortality

A RELATIONSHIP between mortality and metabolic rate may be the basis of a powerful mechanism for the evolution of efficiency in animal locomotion. The ideas presented here were suggested by telemetric observations of brown trout (*Salmo trutta* L.) in their natural environment¹ but may be applicable to a very wide range of animals.

That energetic efficiency is adaptive is an implicit tenet in much recent zoological thought despite the absence of a direct causal link between metabolic efficiency and success in producing offspring. Alexander² describes a simple model for natural selection for efficiency based on the assumption that energy saved in locomotion, for example, is diverted into gonad development so that the efficient genotype produces more offspring. He derives a coefficient of selection of 0.015 for a genotype with non-gonad energy requirements reduced by 1%. This model involves a number of simplifying assumptions and it is clear that increase in gonad products beyond a certain optimum can be disadvantageous³. Selection pressure for efficient energy utilisation may be rather low, any efficiency ratio permitting long term positive energy balance being feasible.

Any animal must function within its 'scope for activity' as defined by Fry⁴. Enforced activity above or below the limits of scope implies certain death. A fish working at moderate power outputs can be assumed to have a small probability of mortality by predation, disease, failure of homeostasis and so on. As the metabolic rate approaches the upper or lower limits of scope so the mortality probability must approach 100%. Metabolic rate (MR) can be normalised to a scale with standard metabolic rate (SMR) as 0 and active metabolic rate (AMR) as 1

$$\frac{MR - SMR}{AMR - SMR} = S$$

Potential mortality per day (M_p) as a function of S probably takes a U-shaped form with M_p equal to 100% at $S < 0$ or $S > 1$ (Fig. 1). Increased time spent at high-activity levels approaching the active metabolic rate may be an important stress and mortality factor in natural populations. Attention has recently been directed to the importance of the cost of reproduction in terms of adult mortality resulting from strenuous activity during the breeding season.⁵

In several marine^{6,7} and freshwater⁸ fish tracking studies it has been shown that high swimming speeds are rare. The total energy requirement for swimming in brown trout has been calculated as 4% of the total ingested energy.^{1,9} This low figure suggests little latitude for fecundity increase by virtue of locomotor energy savings discussed above. Figure 1 shows the typical distribution of metabolic rates of brown trout. Activity in excess of $S = 0.8$ always occupied less than 4% time. The total potential daily mortality (M_{pd}) is equal to the product of potential mortality (M_{ps}) and the time (T_s) at each S level, summed over the whole range of S values:

$$M_{pd} = \sum_{S=0}^{S=1} M_{ps} \times T_s$$

The values of M_{ps} are chosen to give $M_{pd} = 0.1\%$; mortality comparable with that of many natural populations.¹⁰

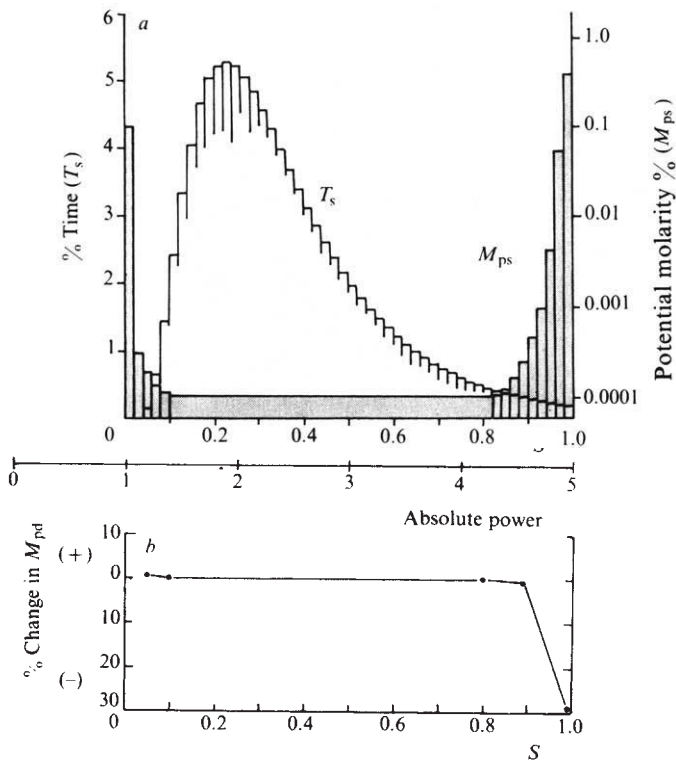


Fig. 1 Activity and mortality in brown trout. *a*, T_s , The typical distribution of time spent at different metabolic rates relative to scope (S , see text). This is based on heart rate telemetry data¹¹ from brown trout in Airthrey Loch, Stirling, Scotland¹. A log-normal distribution was used to fit the curve. $S = 0$ standard metabolic rate. $S = 1$ active metabolic rate. The absolute power or metabolic rate scale is in arbitrary units. M_{ps} , Potential mortality (or failure rate per day) at different values of S . This is a notional distribution based on arguments in the text; no significance is attached to the particular asymmetry or values shown. All calculations are based on the 50 histogram columns shown, each 0.02 S units wide. *b*, Percentage change in total daily potential mortality (M_{pd}) if the total energy expenditure of the fish is reduced by 0.0043% of the total by energy savings at different values of S .

Natural selection consequences of this can be considered. Assume an adaptation appears which reduces drag at a particular speed so that the power required to propel the fish at its maximum cruising speed is reduced by 1%. The time spent at the highest power category can then be replaced by a similar time at a power output 1% lower. In this example the time at $S = 0.99$ is reduced from 0.2% to 0.181% and the time at $S = 0.97$ increases from 0.22 to 0.239. The new value for M_{pd} is 0.0708%: a 29% drop from 0.1% per day. The actual energy saved is only 0.0043% of the total energy expenditure of the fish. If the fish has one year to live until a single spawning the coefficient of selection in favour of the efficient genotype is 0.17 and if it lives to a second spawning a year later the coefficient is 0.244. Thus selection for efficiency can be much more powerful than previously supposed, particularly in long-lived multiple brood animals.

Figure 1*b* shows the change in M_{pd} if the same amount of energy is saved at different levels of S . Reduction of mortality only occurs where the M_{ps} curve has a positive slope. Selection will therefore produce extreme refinement for efficiency at high power outputs but only a small effect at moderate power outputs.

The model in Fig. 1*b* breaks down at low S values since saving energy apparently increases M_{pd} . Energy savings at these S values must consist largely of reduction in standard metabolic rate; that is the $S = 0$ point is shifted to lower absolute power levels. The scope is therefore increased, the crisis of depressed metabolism averted and M_{pd} would decrease.

The penalty of failure to sustain a given activity level is not necessarily death. In the simple case of fish being chased by a

trawl the only escape may be to swim continuously at high speed. Then

$$\frac{\text{Actual mortality}}{\text{Potential mortality}} = C = 1$$

C approaches 1 if: (1) no alternative strategies can be perceived or implemented, alternative resources of food and refuge being important; (2) anaerobic working capacities to escape crises are absent or exhausted; (3) there are no reserves to sustain the animal if it avoids activity and its hazards altogether. The complexities of C should be noted but the main conclusions are not changed. Since C is generally less than 1 the values of M_{ps} in Fig. 1 should be greater than shown. The theory depends critically on the reality of the relationship between M_p and S . Various different shapes of the curve can be envisaged and analogy with engineering reliability theory may be useful in developing the ideas further. Considerations of power capacities and reliability may be more important than energy balance in functional design of animals.

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1. Priede, I. G. & Young, A. H. J. *Fish. Biol.* **10**, 299–318 (1977).
2. Alexander, R. McN. *Functional Design in Fishes* (Hutchinson, London, 1967).
3. Lack, D. *Population Studies of Birds* (Oxford University, 1966).
4. Fry, F. E. J. *Publ. Ont. Fish Res. Lab.* **55** (1947).
5. Stearns, S. V. *Q. Rev. Biol.* **51**, 3–47 (1976).
6. Greer-Walker, M., Mitson, R. B. & Storeton-West, T. J. *Nature* **229**, 196–198 (1971).
7. Hawkins, A. D., MacLennan, D. N., Urquhart, G. G. & Robb, C. J. *Fish Biol.* **6**, 225–236 (1974).
8. Young, A. H., Tytler, P., Holliday, F. G. T. & Macfarlane, A. J. *Fish Biol.* **4**, 57–65 (1972).
9. Holliday, F. G. T., Tytler, P. & Young, A. H. *Proc. R. Soc. Edinb.* **74B**, 315–351 (1974).
10. Frost, W. E. & Brown, M. E. *The Trout* (Collins, Glasgow, 1967).
11. Priede, I. G. & Tytler, P. J. *Fish Biol.* **10**, 231–242 (1977).

Chromosomal recombination between *Rhizobium* species

THE basis for the symbiotic specificity whereby legumes are nodulated only by rhizobia of the appropriate cross-inoculation group must reside in genetic determinants in both host and bacterium. To analyse the bacterial determinants, crosses between rhizobia of different cross-inoculation groups are necessary. We show here that the sex-factor R68.45 (ref. 1), which promotes chromosomal recombination in *Rhizobium leguminosarum*² can also mediate gene transfer amongst strains of *R. leguminosarum*, *R. trifolii* and *R. phaseoli*. These species nodulate peas, clover and French beans respectively; thus there is the possibility of identifying bacterial genes involved in host specificity. The only previous report of interspecific conjugation in *Rhizobium* described the transfer of a plasmid that determined host specificity in *R. trifolii* and *R. phaseoli*³. There have also been reports of transformation of genes, including those determining specificity, between *Rhizobium* species^{4,5}.

In *R. leguminosarum* *rif* (rifamycin resistance) and *str* (streptomycin resistance) alleles are cotransferred at high frequencies². We made use of this finding in a series of preliminary crosses with *R. leguminosarum* strain 6591 (*ura-14 met-14 rif str* R68.45) as donor to strains of *R. trifolii* and *R. phaseoli* from the culture collection at Rothamsted Experimental Station, Harpenden, England. Crosses were plated on minimal medium supplemented with streptomycin (200 mg l⁻¹) and rifamycin (25 mg l⁻¹) to select for cotransfer of the *rif* and *str* alleles from *R.*

leguminosarum. One strain each of *R. trifolii* (6601) and *R. phaseoli* (1231) able to receive and express *R. leguminosarum* *rif* and *str* alleles was chosen for further study.

Crosses were made between multiply-marked R^+ *R. leguminosarum* strains as donors and derivatives of 6601 and 1231 as recipients and between *R. trifolii* (R68.45) or *R. phaseoli* (R68.45) with *R. leguminosarum* as recipient. The frequencies of transfer of single alleles and of co-transfer of non-selected alleles in the crosses were measured. Recombinants differing from each parent by two or more alleles could be distinguished from colonies which had arisen by single mutation of either parent to prototrophy or to antibiotic resistance. No unambiguous recombinants (frequency $< 10^{-8}$) were found when R68.45 was absent from both input strains.

The frequencies of transfer of all the selected markers in either direction between *R. phaseoli* and *R. leguminosarum* and for most of the markers in crosses between *R. trifolii* and *R. leguminosarum* were similar to those found in crosses between strains of *R. leguminosarum*² (about 10^{-6} per recipient). As was found in crosses between *R. leguminosarum* strains, more than 80% of recombinants contained the R plasmid, compared with about 1% for non-recombinant recipients.

In Table 1 the frequencies of cotransfer of various pairs of alleles in interspecific crosses are compared with those found in crosses between strains of *R. leguminosarum*. In the interspecific crosses *rif* and *str* were cotransferred at high frequency whether *R. leguminosarum* was donor or recipient. Moreover *rif* and *rif*⁺, *str* and *str*⁺ alleles were transferred to and from *R. leguminosarum* at approximately equal frequencies (results not shown) indicating that donor genes replaced corresponding genes in the recipient rather than that partial diploids were formed.

In many bacterial species *str* and *spc* (spectinomycin resistance) genes are closely linked⁶⁻⁸. This was true for the *spc* allele of *R. phaseoli* (*spc-19*); it cotransferred with *rif* and *str* alleles to *R. leguminosarum* at high frequency. When selection was made for *rif* and *str* transfer from *R. leguminosarum* to *R. phaseoli* carrying *spc-19* about 90% of the recombinants were spectinomycin sensitive (Table 1), indicating the presence of a hitherto undetected *spc*⁺ allele in *R. leguminosarum* closely linked to the *rif/str* region. A *spc* allele of *R. trifolii* was unlinked to *rif/str* (Table 1) but mapped between *phe-1* and *trp-18*, as did the *spc-4* allele of *R. leguminosarum* (unpublished observations).

With the exception of *spc-19* the cotransfer frequencies of pairs of alleles in crosses between *R. phaseoli* and *R. leguminosarum* were similar to those found in crosses between strains of *R. leguminosarum* (Table 1). This was true also for several pairs of alleles in the crosses between *R. trifolii* and *R. leguminosarum*. The frequencies of co-transfer of *ura-14*⁺ and of *met-12*⁺ with *phe-1*⁺ from *R. trifolii* were, however, about tenfold lower than those found in crosses between strains of *R. leguminosarum* which carried these alleles. Also, the absolute frequencies of transfer of *ura-14*⁺ and *met-12*⁺ in the *R. trifolii* × *R. leguminosarum* crosses were about ten times lower than those found for other markers. We do not know whether these alleles are located at another region in *R. trifolii* or whether they do map at the same region as in *R. leguminosarum*, but only that the frequency of recombination in this region is reduced in these interspecific crosses.

If we assume that most of the genes between a pair of alleles were also transferred, integrated into the recipient chromosome and expressed, any essential genes within that region in the recipient must be functionally replaceable by corresponding alleles in the donor. This reinforces the idea that the sequence of genes in the three *Rhizobium* species is very similar. This should greatly facilitate gene mapping in these and perhaps in other species of *Rhizobium*.

To determine whether any of the interspecific recombinants had altered host specificity, individual recombinants inheriting at least two donor alleles were used to inoculate plants; the recombinants between *R. trifolii* and *R. leguminosarum* were tested on peas (*Pisum sativum* cv. Dark Skinned Perfection) and white clover (*Trifolium repens* cv. S100 in the collection at the Welsh Plant Breeding Station, Aberystwyth, Wales) and the recombinants between *R. phaseoli* and *R. leguminosarum* were tested only on peas. Although the frequency of multiple crossover events in recombination is unknown, it is likely that most recombinants received the majority of the genes lying between the two selected alleles. This assumption is supported by the finding that in a cross between *R. trifolii* strain 6647 (*rif spc-20* R68.45) and *R. leguminosarum* strain 1156 (*phe-1 trp-18 str*) all *phe-1*⁺ *trp-18*⁺ recombinants also received the intervening *spc* allele from *R. trifolii*. The recombinants all retained the specificity of the recipient and did not acquire that of the donor (Table 2). Thus it seems that genes determining specificity did not reside in the transferred fragments, or, if they did, that they were not expressed in the heterospecific recipient.

Most studies on interspecific and intergeneric recombination have been done on members of the *Enterobacteriaceae*⁹. The main feature of such crosses is the lowered recombination frequency, although in some cases gene sequences are very similar, as in *Escherichia coli* and *Salmonella typhimurium*. A possible explanation for the general lack of reduction in recombination frequencies in the *Rhizobium* interspecific crosses reported here is that the strains were very closely related and had very similar DNA sequences. Another explanation is that recombination mediated by R68.45 is less dependent on DNA homology than is Hfr-mediated recombination in the *Enterobacteriaceae*. On the basis of numerical taxonomy *R. leguminosarum*, *R. trifolii* and *R. phaseoli* are closely related¹⁰; this was the reason for their choice in this study. Preliminary evidence suggests,

Table 1 Frequencies of cotransfer of pairs of alleles between *R. phaseoli*, *R. trifolii* and *R. leguminosarum*

Alleles cotransferred	a, <i>R. leguminosarum</i> as donor* Mean cotransfer frequency (%) in recipient species		
	<i>R. leguminosarum</i>	<i>R. phaseoli</i>	<i>R. trifolii</i>
<i>rif, str</i>	80	79	85
<i>rif, spc</i> ⁺	NT	89†	< 0.7‡
<i>str, rib-2</i>	7	9	13
<i>str, cys-8</i>	7	1	3
Alleles cotransferred	b, <i>R. leguminosarum</i> as recipient Donor species		
	<i>R. leguminosarum</i>	<i>R. phaseoli</i>	<i>R. trifolii</i>
<i>rif, str</i> ⁺	80	94	83
<i>rif, spc</i>	< 0.7	83†	< 0.7‡
<i>spc, str</i> ⁺	< 0.7	88†	< 0.7‡
<i>str, rib-2</i> ⁺	7	NT	3
<i>phe-1</i> ⁺ , <i>trp-18</i> ⁺	32	34	32
<i>phe-1</i> ⁺ , <i>met-12</i> ⁺	19	8	< 1
<i>phe-1</i> ⁺ , <i>ura-14</i> ⁺	13	14	1
<i>rib-2</i> ⁺ , <i>ade-27</i> ⁺	7	14	11
<i>ura-14</i> ⁺ , <i>trp-18</i> ⁺	1	1	1
<i>spc, trp-18</i> ⁺	NT	< 0.7†	75†
<i>spc, phe-1</i> ⁺	NT	< 0.7†	41‡
<i>spc, ura-14</i> ⁺	NT	< 0.7†	6‡
<i>spc, met-14</i> ⁺	NT	< 0.7†	32‡

*Donor/recipient refers to those parental strains in the crosses which respectively carried and did not carry the R-plasmid R68.45.

†*R. phaseoli spc-19* was used.

‡*R. trifolii spc-20* was used.

NT, Not tested

The genetically marked strains of *R. leguminosarum* were derived from strain 300 (ref. 12). The *R. trifolii* and *R. phaseoli* strains were derived from strains 12 and 3644 of the Rothamsted culture collection respectively. Methods for mutant isolation, media and culture conditions have been described¹⁴, as have the methods for the analysis of linkage in crosses². In this work 150 colonies were picked from each selective medium and analysed for non-selected markers.

Table 2 Nodulating ability of interspecific recombinants

Donor species	Recipient species	Alleles cotransferred	Number of recombinants tested	Number of plants with nodule	
				Peas	Clover
<i>R. leguminosarum</i>	<i>R. phaseoli</i>	<i>rif</i> , <i>str</i>	15	0	—
<i>R. leguminosarum</i>	<i>R. trifolii</i>	<i>rif</i> , <i>str</i>	37	0	37
<i>R. leguminosarum</i>	<i>R. trifolii</i>	<i>str</i> , <i>trp</i> -14	3	0	3
<i>R. leguminosarum</i>	<i>R. trifolii</i>	<i>str</i> , <i>rib</i> -2	11	0	11
<i>R. leguminosarum</i>	<i>R. phaseoli</i>	<i>str</i> , <i>rib</i> -2	11	0	—
<i>R. trifolii</i>	<i>R. leguminosarum</i>	<i>met</i> -14 ⁺ , <i>spc</i> -20	9	9	0
<i>R. trifolii</i>	<i>R. leguminosarum</i>	<i>phe</i> -1 ⁺ , <i>trp</i> -18 ⁺ , <i>spc</i> -20	15	15	0
<i>R. trifolii</i>	<i>R. leguminosarum</i>	<i>ade</i> -27 ⁺ , <i>rib</i> -2 ⁺	11	11	0
<i>R. trifolii</i>	<i>R. leguminosarum</i>	<i>trp</i> -16 ⁺ , <i>ura</i> -14 ⁺ , <i>spc</i> -20	4	4	0

Peas were inoculated with the interspecific recombinants as described¹⁴. Clover seeds were surface sterilised with sodium hypochlorite, transferred to slopes of water agar in test tubes and nitrogen-free medium¹⁵ containing the inoculum was added. The clover was grown at 26 °C under fluorescent lights. Clover plants were scored for nodulation after 2–3 weeks and peas after 3–4 weeks. Two or three plants were used for each recombinant tested.

however, that *rif* and *str* alleles of *R. leguminosarum* may be transferred by R68.45-mediated recombination to a strain of the apparently more distantly related species¹⁰, *R. meliloti* (unpublished observations). It is relevant that in certain conditions different *Rhizobium* species may occupy the same nodule in substantial numbers¹¹. Such a situation might provide an opportunity for gene exchange in the wild if a sex factor were present in one of the strains.

The ability of fairly large regions of the chromosome to be transferred between strains within a *Rhizobium* species^{2,12} allows contemplation of the possibility of a rhizobium breeding programme whereby recombinants containing favourable attributes from different parental strains (for example competitiveness for nodulation, high levels of nitrogen fixation, tolerance to certain environments) could be constructed. Such strains might prove superior to the parentals for use as inocula. The finding of interspecific recombination means that the gene pool for the formation of such recombinants need not be restricted to the variation encountered within a single species.

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- ¹ Haas, D., & Holloway, B. W. *Molec. gen. Genet.* **144**, 243–251 (1976).
- ² Beringer, J. E. & Hopwood, D. A. *Nature* **264**, 291–293 (1976).
- ³ Higashi, S. J. *gen. appl. Microbiol.* **13**, 391–403 (1967).
- ⁴ Balassa, R. *Nature* **188**, 246–247 (1960).
- ⁵ Lange, R. T., & Alexander, M. *Can. J. Microbiol.* **7**, 959–961 (1961).
- ⁶ Young, F. E., & Wilson, G. A. in *Handbook of Genetics* **1**, 69–114, (Plenum, New York, 1974).
- ⁷ Bachmann, B. J., Low, K. B. & Taylor, A. L. *Bact. Rev.* **40**, 116–167 (1976).
- ⁸ Hopwood, D. A., Chater, K. F., Dowding, J. E. & Vivian, A. *Bact. Rev.* **37**, 371–405 (1973).
- ⁹ Sanderson, K. E. *A. Rev. Microbiol.* **30**, 327–349 (1976).
- ¹⁰ Graham, P. H. *J. gen. Microbiol.* **35**, 511–517 (1964).
- ¹¹ Johnston, A. W. B., & Beringer, J. E. *Nature* **263**, 502–504 (1976).
- ¹² Johnston, A. W. B., & Beringer, J. E. *J. gen. Microbiol.* **87**, 343–350 (1975).
- ¹³ Meade, H., & Signer, E. *Proc. natn. Acad. Sci. U.S.A.*, (in the press).
- ¹⁴ Beringer, J. E. *J. gen. Microbiol.* **84**, 188–198 (1974).
- ¹⁵ Cartwright, P. M. *Ann. Bot.* **31**, 309–321 (1967).

acquires its adult functional capacities over a protracted span of postnatal development^{1–3}. Most of this evidence, however, has come from studies involving surgical ablation. The study of cerebral development in brain-damaged subjects is complicated by anterograde and retrograde degenerative changes, as well as by the possibility of compensatory neural reorganisation which could occur during the weeks or months between surgical ablation and assessment of behavioural performance⁴. It is now possible to study the functions of specific brain sites without producing permanent brain damage by localised cortical cooling, a method which allows reversible inactivation of restricted regions of cortex in otherwise normal animals. The latter are able to serve as their own controls before and after the episodes of cortical hypothermia^{5–7}. This method has previously been used only in research on mature animals. In this study, local hypothermia was produced for the first time in the dorsolateral prefrontal cortex of young, developing rhesus monkeys during repeated assessment of their performance on a delayed-response task. Such performance in adult monkeys is known to be dependent on the integrity of that region⁸. Our findings provide direct evidence that part of the dorsolateral prefrontal association cortex does not become functionally mature until at least 34–36 month of age, that is, close to sexual maturity in this species.

Seven rhesus monkeys (*Macaca mulatta*) aged 9–36 months were trained to perform a 12-s, two-choice delayed response task. After each monkey reached a criterion performance of 90% correct on four consecutive days, cooling chambers were implanted over a portion of the dorsolateral prefrontal cortex^{5,8} in each hemisphere under pentobarbital anaesthesia. The disk-shaped stainless steel cooling probes (12.5 mm diameter) were implanted epidurally, centered 4 mm above the midpoint of the principal sulcus; the dura remained intact except for two small openings through which insulated bead thermistors were inserted to monitor the subdural temperature. Experimental testing began 1 week after recovery from surgery and consisted of repeated sessions in which the subject's delayed-response performance was assessed before, during and after cooling to 20 °C. Each monkey received 100–200 trials in each of the three conditions (precool, cool, postcool) over the course of 10 sessions.

Intracerebral temperature gradients measured during cooling at terminal stages of the experiment revealed that the areas cooled to 20 °C in all brains was limited to tissue directly beneath the probe and included only the superficial banks of the principal sulcus. The depths of that sulcus remained within 2–3 °C of the normal core temperature. In addition there was no significant hypothermia beyond the boundaries and subjacent white matter of the dorsolateral prefrontal cortex. It should be emphasised that no

Maturation of prefrontal cortex in the monkey revealed by local reversible cryogenic depression

THE emergence of increasingly complex cognitive functions with age is widely assumed to reflect in part the maturation of connections within the central nervous system, but the neural bases of cognitive development have resisted identification. There is some evidence to indicate that the dorsolateral prefrontal association cortex in monkeys

significant variation was found in the size or locus of the area cooled in relation to age (Fig. 1). In contrast, the behavioural results were clearly age dependent, as shown in Fig. 2, where the monkeys have been grouped according to age. Dorsolateral prefrontal cooling did not impair delayed-response performance in monkeys 9–16 months of age (Group I). This group made a small number of errors during cooling, but this figure did not differ significantly from the number of errors made during postcool trials. A small but significant increment (7%) in errors due to cooling was first observed in monkeys 19–31 months of age (Group II), but cooling-induced deficits comparable in magnitude (23%) with those seen in adult monkeys^{3,6} did not appear until 34–36 months of age (Group III). The percentage of cooling-induced errors exhibited by Group III monkeys was on the average 2.5-fold higher than that exhibited by the monkeys in Group II.

The pattern of increasing sensitivity to dorsolateral prefrontal cooling, apparent in the comparisons among groups of monkeys at different ages, is also demonstrable in the ontogenetic history of individual monkeys. Thus, one monkey (Fig. 3) manifested a significant but relatively small increase in errors during prefrontal cooling at the age of 31 months, whereas 3 months later this same animal exhibited a 2.5-fold increment in the percentage of errors during cooling, while its control performance during this same interval showed a slight improvement.

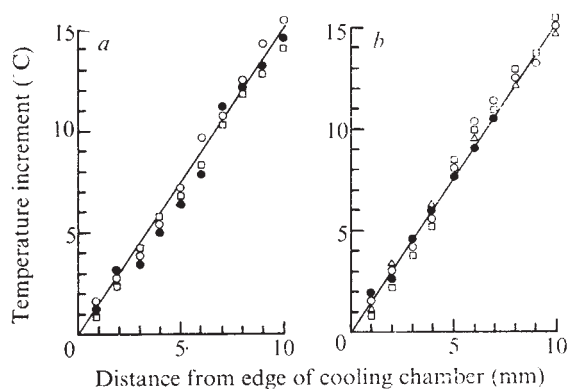


Fig. 1 Intracerebral temperature gradients in two planes, determined with cortical surface beneath the cooling chamber maintained at 20 °C. Shown are the data from four animals aged 9 (□), 18 (△), 34 (●) and 36 (○) months, respectively. *a*, Gradient recorded tangential to the cortical surface. Least square line through data has slope of 1.57 °C mm⁻¹ and correlation coefficient of 0.989. *b*, Gradient recorded perpendicular to cortical surface. Least square line has slope of 1.51 °C mm⁻¹ and correlation coefficient of 0.995. Since the brains of the youngest and oldest monkeys differed in size by no more than 5%, and the probe placement was identical in all, there was no significant difference with age in the amount or location of the tissue cooled.

The finding that prefrontal cooling did not induce deficits in the Group I monkeys is in agreement with previous reports that even complete resections of dorsolateral prefrontal cortex in infancy do not produce impairments on delayed response¹⁻³. The 23% performance decrement observed in the oldest (Group III) monkeys during cooling is, however, substantially less than the nearly 50% decrement usually seen in adult monkeys with prefrontal lesions. This discrepancy is most likely due to the fact that the cortex in the depths of the principal sulcus, normally included in dorsolateral surgical ablations, was not subjected to cryogenic depression. But, as the magnitude of the cooling-induced impairment in the Group III monkeys is nearly identical to that reported in independent studies of adult monkeys carried out with comparable methods^{3,6}, it seems that the Group III monkeys represent the age at which the dorsolateral cortex, exclusive of the depth of the principal sulcus, reaches functional maturity.

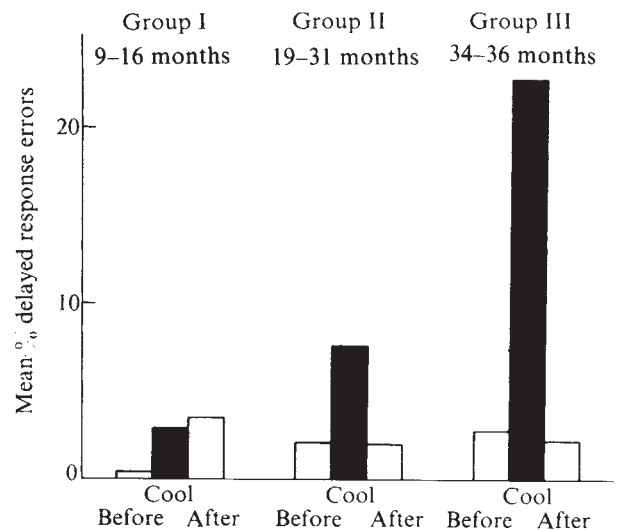
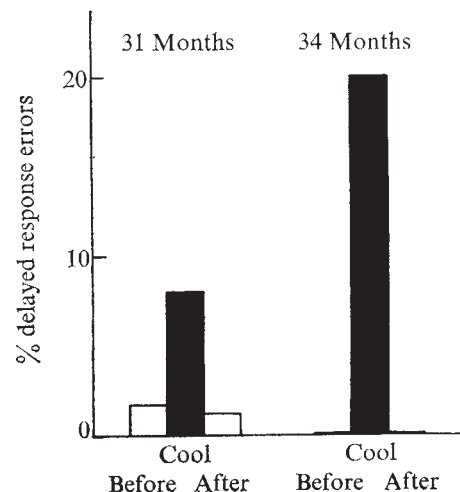


Fig. 2 Delayed response (DR) performance in relation to cryogenic depression of the dorsolateral prefrontal cortex (DLC). Mean % errors on DR trials presented before, during and after DLC cooling for groups of monkeys in the age spans indicated. % DR errors for each animal and condition were subjected to an arcsin transformation, and the transformed data underwent a 3 (age groups) × 3 (DLC cooling condition) factorial analysis of variance with repeated measures on the last-named variable. The main effect for DLC cooling condition was significant ($F(2,10) = 28.1$, $P < 0.001$), as was the age × DLC cooling condition interaction ($F(4,10) = 8.8$, $P < 0.005$). Comparisons of mean DR errors in the different DLC conditions within groups were made using Tukey's (*a*) test with the critical level of $P < 0.01$. Both Groups II (ages 19–31 months, $n = 3$) and III (ages 34–36 months, $n = 2$) made significantly more errors during cooling than either before or after while their performance under the two latter conditions did not differ significantly. In contrast, for Group I (ages 9–16 months, $n = 3$), there were no significant differences in performance among any of the three conditions.

Thus, the present results indicate that a considerable portion of the dorsolateral prefrontal cortex does not participate significantly in the mediation of delayed-response performance during the first 16 months of postnatal life; subsequently, this region participates only minimally until, by 36 months of age, the process of maturation seems to be essentially complete. Previous evidence from ablation studies for the protracted maturation of the dorsolateral prefrontal cortex was open to the alternative explanation that early lesions of this area had failed to result

Fig. 3 % Errors on DR trials presented before, during and after DLC cooling in a single monkey tested at two different stages of development.



in deficits months after surgery because there had been time for recovery of function through compensatory neural reorganisation⁴. This explanation now seems improbable because prefrontal cooling did not produce delayed-response deficits in young, non-lesioned monkeys. In these animals neural reorganisation should not be a factor unless it were assumed that such a process could be stimulated by cortical hypothermia within the course of a testing session.

The lack of dependence on the dorsolateral prefrontal cortex for delayed response behaviour in young monkeys, shown now both in studies of surgical and functional ablation, contrasts with the dependence for such behaviour, from infancy through sexual maturity, on certain brain regions other than the dorsolateral prefrontal cortex, such as the orbital prefrontal cortex^{1,9}, the anterodorsal caudate nucleus¹⁰ and the mediodorsal nucleus^{4,11}. Our results suggest that the dorsolateral prefrontal cortex assumes its mature functions by augmenting rather than replacing these other antecedent neural mechanisms, since there is no evidence of disturbance in the basic ability to perform delayed response during the transition from minimal to maximal dependence upon the dorsolateral prefrontal cortex.

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- 1 Goldman, P. S. *Expl Neurol.* 32, 366-387 (1971).
- 2 Harlow, H. F., Akert, K. & Schiltz, K. A., in *The Frontal Granular Cortex and Behavior* (eds Warren, J. M. & Akert, K.) 126-145 (McGraw-Hill, New York, 1964).
- 3 Tucker, T. J. & Kling, A. *Brain Res.* 5, 377-389 (1967).
- 4 Goldman, P. S. in *CNS Plasticity and Recovery of Function* (eds Stein, D. G., Rosen, J. & Butters, N.) 149-174 (Academic, New York, 1974).
- 5 Alexander, G. E. & Fuster, J. M. *Brain Res.* 61, 93-105 (1973).
- 6 Fuster, J. M. & Alexander, G. E. *Brain Res.* 20, 85-90 (1970).
- 7 Bauer, R. H. & Fuster, J. M. *J. comp. Physiol. Psychol.* 90, 293-302 (1976).
- 8 Goldman, P. S., Rosvold, H. E., Vest, B. & Galkin, T. W. *J. comp. Physiol. Psychol.* 77, 212-220 (1971).
- 9 Miller, E. A., Goldman, P. S. & Rosvold, H. E. *Science* 182, 304-306 (1973).
- 10 Goldman, P. S. & Rosvold, H. E. *Brain Res.* 43, 53-66 (1972).
- 11 Schulman, S. *Archs Neurol.* 11, 477-499 (1964).
- 12 Teuber, H. L. & Rudel, R. *Devl Med. Child Neurol.* 4, 3-20 (1962).
- 13 Basser, L. S. *Brain* 85, 427-460 (1962).

Gene dosage for isocitrate dehydrogenase in mouse embryos trisomic for chromosome 1

It is generally assumed that changes in chromosome number are reflected in gene dosage effects for loci located in the extra or missing chromosomes. Although such effects have been well substantiated in *Drosophila*¹, data for mammalian aneuploidy are scarce. Quantitative gene dosage effects have been demonstrated for several X-linked enzymes in oocytes of mice with X-chromosome aneuploidy^{2,3} and for malic enzyme in mice with partial trisomy of chromosome 7 (ref. 4). In man, similar effects for superoxide dismutase-1 (W. W. Feaster, L. W. Kwok and C.J.E., unpublished and ref. 5), adenine phosphoribosyltransferase⁶, purine nucleoside phosphorylase⁷ and erythrocyte acid phosphatase⁸ have been observed in cells aneuploid for chromosomes 21, 16, 14 and 2, respectively. Aneuploidy for chromosome 21 also affects responsiveness to interferon⁹, and XYY cells have been claimed to have increased amounts of H-Y surface antigen¹⁰. Since the detection of more than 30 metacentric (Robertsonian translocation) chromosomes in wild and laboratory strains^{11,12} and the development of a mating scheme which utilises such translocations for the production of aneuploid embryos in relatively high frequencies^{13,14}, the mouse has become particularly useful for gene dosage studies. We have used



Fig. 1 Metaphase spreads from membranes of trisomic mouse embryos prepared as described by Evans *et al.*¹⁵. a, Trisomy 1; b, trisomy 12. The two metacentric chromosomes indicative of the trisomic state are indicated by arrows in each spread.

this system to demonstrate the dosage effect for supernatant isocitrate dehydrogenase (Id-1) which results from trisomy for chromosome 1.

Males doubly heterozygous for two different metacentric chromosomes which contain chromosome 1, Rb1(1.3)Bnr and Rb10(1.10)Bnr, were obtained by mating animals homozygous for Rb1 with Rb10 homozygotes. To produce embryos trisomic for chromosome 1, heterozygous males were mated with mature white Swiss (WS) females (Simonsen Laboratories), and the time of mating was confirmed by observation of vaginal plugs. For control experiments, embryos trisomic for chromosome 12 or chromosome 19 were generated by using Rb5(8.12)Bnr/Rb9(4.12)Bnr and Rb1(5.19)Wh/Rb163(9.19)H heterozygotes, respectively. These aneuploid embryos are known to be viable until day 14 or 15 of gestation¹⁵. The pregnant females were killed on day 12 or 13, and the embryos (with membranes) were removed. The chromosome constitution of each embryo was determined by examination of metaphase spreads made after short term incubation of the membranes in colcemid as described before¹⁵. Trisomic embryos were identified by their two metacentric chromosomes and total of 41 chromosome arms. Because monosomic embryos die at or shortly after implantation¹⁶, all other karyotypes with only one metacentric chromosome are euploid. As Table 1 shows, between 15 and 42% of the embryos were trisomic. Representative metaphases from trisomic embryos are shown in Fig. 1.

While the membranes were being prepared for chromosome analysis, the individual whole embryos were homogenised and analysed for enzyme activity spectrophotometrically and, when appropriate, by starch gel electrophoresis. The results of assays for Id-1 activity in embryos trisomic for chromosome 1, 12 or 19, and for glucose-6-phosphate dehydrogenase (G6PD, X-linked) and 6-phosphogluconate dehydrogenase (*Pgd*, chromosome 4) in trisomy 1 embryos are summarised in Table 1. Control embryos were the karyotypically normal embryos from the same litters as the trisomics. Trisomy 1 was associated with 1.53 ± 0.04 (s.d.) times the control activity of Id-1, but neither G6PD or *Pgd* activity was significantly different from the control. Thus trisomy 1, with three chromosome 1 instead of two, results in the expected 3:2 increase in activity of chromosome 1-determined Id-1, but not of enzymes coded by other chromosomes; trisomy for either of two other chromosomes does not affect the activity of Id-1.

Given the observed Id-1 activity in trisomy 1, it would

Table 1 Enzyme activities in trisomic embryos

Mating	Chromosome	Trisomy Frequency	Enzyme activity in trisomic embryos (ratio to normal)		
			Id-1	G6PD	PGD
WS × Rb1Bnr/Rb10Bnr	1	14/91 (15%)	1.53 ± 0.04 (6)	0.96 ± 0.05 (5)	0.94 ± 0.26 (5)
WS × Rb5Bnr/Rb9Bnr	12	7/23 (30%)	1.06 ± 0.05 (4)		
WS × Rb1Wh/Rb163H	19	11/26 (42%)	0.98 ± 0.06 (7)		

Results are expressed as mean $\pm$ s.d. of the ratios of specific activities based on soluble protein. The number of determinations is shown in parentheses. Each embryo was individually homogenised in a plastic tube (6 × 50 mm) with a glass homogeniser in 0.05 ml 0.25 M sucrose in 0.01 M phosphate buffer, pH 7.0. Another 0.05 ml sucrose-phosphate was added, and the homogenate was then centrifuged at 27,000g for 20 min at 4 °C. The supernatant fluids were used for enzyme assay and protein determinations. Id-1 was assayed as described by Lowry and Passonneau¹⁸, and G6PD and PGD as described by Glock and McLean¹⁹.

be expected that each of the three genes for Id-1 contributes equally to the total product. To test this directly, the enzyme produced by trisomic and normal embryos was subjected to starch gel electrophoresis. Since Rb1Bnr carries the *b* (fast) allele at *Id-1* and Rb10 the *a* (slow) allele, the Rb1/Rb10 heterozygote is electrophoretically *ab*. Depending on the allele contributed by the Swiss female, which can be either *a* or *b*, the genotype for the *Id-1* of the trisomic embryos will be either *aab* or *abb*. Because *Id-1* is a dimer, the possible enzyme products are *aa*, *ab* and *bb*. If each gene is functionally equal, the expected ratios of the three are 4:4:1 (if the embryo is *aab*) or 1:4:4 (if *abb*). Normal embryos will be *aa*, *bb* or *ab*, and the electrophoretic patterns will show either pure *aa*, pure *bb*, or *aa*, *ab* and *bb* in a 1:2:1 ratio. As Fig. 2 shows, patterns compatible with the expected trisomic and normal patterns were observed. Furthermore, no patterns suggestive of the presence of three operative *Id-1* genes were observed with embryos trisomic for chromosome 12. Thus the biochemical data are consistent with an exact and specific dosage effect for *Id-1* in trisomy 1.

With the large number of mouse translocations which have been identified and which are being placed on inbred backgrounds^{13,17}, it will soon be possible to produce and investigate embryos and cell lines derived from them which are trisomic for most of the mouse autosomes. This approach, of which this report is an example, should permit a systematic and refined examination of the molecular and developmental consequences of chromosome imbalance.

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- Lindsley, D. L. *et al. Genetics* **71**, 157–184 (1972).
- Epstein, C. J. *Science* **175**, 1467–1468 (1972).
- Kozak L. P., McLean, G. K. & Eicher, E. M. *Biochem. Genet.* **11**, 41–47 (1974).
- Eicher, E. M. & Coleman, D. L. *Genetics* (in the press).
- Sinet, P. M., Lavelle, F., Michelson, A. M. & Jerome, H. *Biochem. biophys. Res. Commun.* **67**, 904–909 (1975).
- Marimo, B. & Giannelli, F. *Nature* **256**, 204–206 (1975).
- George, D. L. & Francke, U. *Science* **194**, 851–852 (1976).
- Magenis, R. E. *et al. Proc. natn. Acad. Sci. U.S.A.* **72**, 4526–4530 (1975).
- Epstein, L. B. & Epstein, C. J. *J. Infect. Dis.* **133**, suppl. A56 (1976).
- Wachtel, S. S. *et al. New Engl. J. Med.* **293**, 1070–1072 (1972).
- Gropp, A., Winking, H., Zech, L. & Müller, H. J. *Chromosoma, Berl.* **39**, 265–288 (1972).
- Capanna, E., Gropp, A., Winking, H., Noack, G. & Civitelli, M.-V. *Chromosoma, Berl.* (in the press).
- Gropp, A., Kolbus, U. & Giers, D. *Cytogenet. Cell Genet.* **14**, 42–62 (1975).
- White, B. J., Tjio, T. H., Vandewater, L. C. & Crandall, C. *Cytogenet. Cell Genet.* **13**, 217–231 (1974).
- Evans, E. P., Burtenshaw, M. D. & Ford, C. E. *Stain Tech.* **47**, 229–234 (1972).
- Ford, C. E. in *Edinburgh Symposium on the Genetics of the Spermatozoon* (eds R. A. Beatty & S. Gluecksohn-Waelsch) 359–368 (Edinburgh, 1971).
- Cattanach, B. M., Williams, C. E. & Bailey, H. *Cytogenetics* **11**, 412–423 (1972).
- Lowry, O. H. & Passonneau, J. V. *A Flexible System of Enzymatic Analysis*, 95 (Academic, New York, 1972).
- Glock, G. E. & McLean, P. *Biochem. J.* **55**, 400–408 (1953).
- Ruddle, F. H. & Nichols, E. A. *In Vitro* **7**, 120–131 (1971).
- Henderson, N. S. *J. exp. Zool.* **158**, 263–273 (1975).

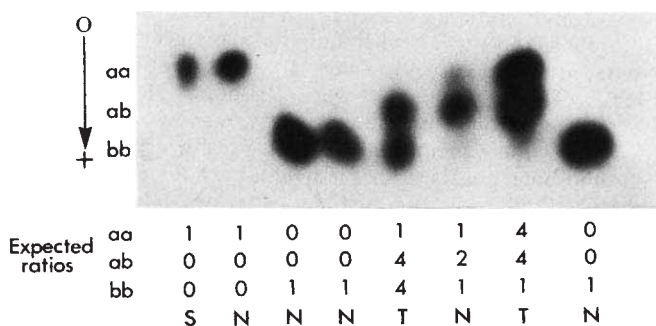


Fig. 2 Electrophoretic patterns of *Id-1* from normal (N) embryos and embryos trisomic (T) for chromosome 1. Electrophoresis was carried out in an 11% vertical starch gel for 20 h at 4 °C at a constant voltage of 120 V using the citrate-phosphate buffer system (No. 3) of Ruddle and Nichols²⁰. The gels were stained as described by Henderson²¹. The origin (O) is at the top and the samples were run to the anode (+). The two trisomic embryos show patterns compatible with the expected 1:4:4 and 4:4:1 ratios for the *abb* and *aab* genotypes, respectively. The five normal embryos display the pure *a*, pure *b*, or 1:2:1 patterns compatible with *aa*, *bb* or *ab* genotypes, respectively. The standard (S) for the *a* (slow) allele was obtained from a liver homogenate from a BALB/c mouse.

Heparin-like inhibitor of blood coagulation in normal newborn

THE level of the blood coagulation factors in the newborn is known to be markedly lower than that in the adult. Generally this is attributed to a lack of synthetic capacity of the liver and a deficiency in vitamin K. We describe here a re-investigation of this problem making use of recently developed methods that determine both the coagulation factors and the abnormal proteins induced by vitamin K absence (PIVKAs). We found low coagulation factor activity in the newborn but no evidence for vitamin K deficiency. In more than half the samples a hitherto unrecognised inhibitor of blood coagulation could be demonstrated. At 3 d of age this inhibitor was found in all babies investigated. On the basis of its properties in blood coagulation tests the inhibitor could not be distinguished from heparin.

In 43 healthy newborns a 20-cm piece of umbilical cord was clamped directly after birth and 9 ml of blood was withdrawn by puncture from the vena umbilicalis into a polypropylene syringe containing 1 ml of a solution containing HEPES 0.3 M, trisodium citrate 0.1 M, sodium azide 15 mM, Trasylol (Bayer) 50 U, pH 7.3. This mixture effec-

tively prevents fibrinolysis. Plasma was obtained by centrifugation and spun platelet poor by a second centrifugation (30 min 4 °C, 20,000g). It was frozen in 1-ml aliquots and stored at -20 °C until further use. In these plasmas we determined clotting factors V, VII, and X and prothrombin by a one-stage procedure and prothrombin also by a two-stage procedure (Table 1). Experimental methods were as described in ref. 1.

In 27 of the 43 plasmas the disappearance rate of thrombin in the two-stage prothrombin assay was significantly (>5 times) greater than in normal human adult plasma. In the 16 others it was no more than 1.8 times greater than normal. On the basis of this observation we divided the plasma in an inactivated and a non-inactivated group. We found no significant differences in antithrombin III, α_1 -antitrypsin, α_2 -macroglobulin, antiplasmin, C_1 -esterase inhibitor and inter- α -trypsin inhibitor activities between inactivated and non-inactivated plasma samples: we therefore conclude that none of these proteins are responsible for the inactivation. No fibrinogen degradation products could be detected in either of the plasmas. Pooled inactivated and pooled non-inactivated plasma was diluted 20-fold with distilled water; isoelectric precipitation at pH 5.2 yielded a fraction containing prothrombin but no thrombin inhibitors. In such fractions made from both plasma types, thrombin generated normally and was not inactivated. In the presence of normal plasma the thrombin thus generated inactivated normally. This excluded the possibility that the rapid inactivation was due to an abnormality of the (pro)thrombin molecule in the inactivated plasma.

Both procoagulants and thrombin inactivating proteins in inactivated plasma were precipitated by phosphotungstic acid (1/3 volume of 10% phosphotungstic acid in 0.67 N H_2SO_4). After neutralisation and removal of the phosphotungstic acid, the resulting fraction accelerated thrombin inactivation in normal plasma by a factor five to seven, and lost its inactivation enhancing properties on addition of 12 $\mu g\ ml^{-1}$ protamine sulphate. No such fraction could be obtained from the non-inactivated plasma. Inactivation of thrombin in inactivated plasma could be prevented by addition of 14 $\mu g\ ml^{-1}$ of protamine sulphate whereas such addition had little or no effect on non-inactivated plasma.

Heparin is known to potentiate thrombin inactivation by antithrombin III, to be neutralised by protamine sulphate and not to be precipitated by phosphotungstic acid².

We conclude that the inactivated plasma contains heparin or a substance with heparin-like properties. The source of this substance is presumably in the newborn. It could not be demonstrated in maternal blood or in the perfused placenta. Five children who did not show enhanced inactivation in

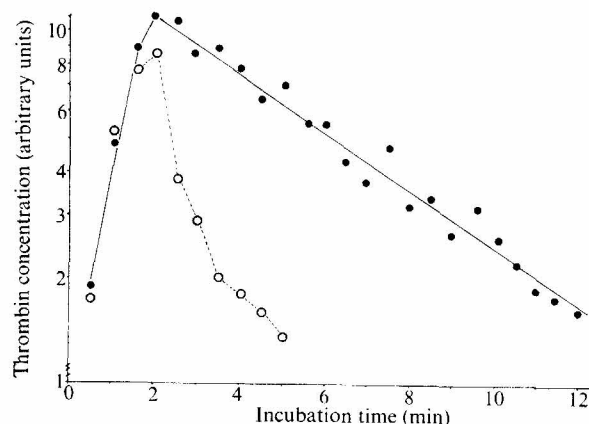


Fig. 1 Thrombin generation and breakdown in pooled newborn plasmas with (○) and without (●) inactivating substance. Reaction mixture: 0.2 ml plasma, 0.1 ml human brain thromboplastin, 1.7 ml Michaelis buffer pH 7.2, 1 ml $CaCl_2$ 33 mM. At the times indicated 0.1 ml of the reaction mixture was subsampled into 0.1 ml of $BaSO_4$ adsorbed plasma diluted 1:5 with Michaelis buffer (pH 7.2). From the clotting time obtained the thrombin content was calculated¹.

the cord blood did show this activity in blood taken from the vena cubiti on day 3 of life. The 11 others from the non-inactivated group were not tested.

Presumably this heparin-like substance is produced in organs that are hardly perfused before birth, but will have an active circulation afterwards (that is, the lungs). It may serve to prevent thrombosis in these organs. In this context it is interesting to note that complete deficiency of antithrombin has never been observed although several series of (possibly heterozygous) patients with about a half normal amount of antithrombin III have been reported³. Complete absence of antithrombin III which would prevent the action of the heparin-like substance might be a lethal condition.

We found no differences between the inactivated and non-inactivated groups in any of the coagulation factors tested. More interesting, there was no discrepancy between the prothrombin contents found with the one-stage and the two-stage assay (Table 1). This indicates that no proteins induced by vitamin K absence (PIVKAs) are present in the plasma of the newborn⁴. This is corroborated by the fact that neither in the staphylocoagulase test nor in the Echis Carinatus test nor in a one-dimensional immuno electrophoresis an excess of prothrombin activity was observed whereas the PIVKA when present would co-estimate in these tests (Table 1, ref. 1).

We concluded that, contrary to current beliefs, the newborn has no vitamin K deficiency but is 'anticoagulated' by heparin or a heparin-like substance.

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¹ Bas, B. M., Muller, A. D. & van der Voort-Beelen, J. M. *J. molec. Med.* 1, 65-72 (1975).

² Gastpar, H. *Thrombos. Diathes. haemorrh.* Suppl. 16, (1965).

³ Van der Meer, J., Stoepman-v. Dalen, E. A. & Jansen, J. M. S. *J. clin. Path.* 26, 532-538 (1973).

⁴ Hemker, H. C., Muller, A. D. & Loeliger, E. A. *Thrombos. Diathes. haemorrh.* 23, 633-637 (1970).

Table 1 Determination of clotting factors in cord blood

Factor	Method	Blood activity (%)	
		I	Non-I
Antithrombin III	CIE	62±8	61±12
α_1 -Antitrypsin	CIE	74±12	81±15
α_2 -Macroglobulin	CIE	104±20	110±16
Antiplasmin	CIE	49±9	49±8
C_1 esterase inhibitor	CIE	53±11	58±12
Inter- α -trypsin inhibitor	CIE	64±14	65±13
II	one-stage	51±12	47±10
II	two-stage	48±12	47±11
II	Echis Carinatus venom	54±11	49±9
II	staphylocoagulase	48±8	48±10
II	CIE	53±15	50±16
V	one-stage		
VII	one-stage	43±12	44±10
X	one-stage	31±15	30±7

Values are means $\pm$ s.d. of the blood activities as % of pooled blood from 30 normal adults. I, group showing rapid thrombin inactivation ($n=27$); Non-I, group showing normal thrombin inactivation ($n=16$). CIE, crossed immunoelectrophoresis.

Abrogation of reagenic antibodies with modified allergens

ALLERGIC diseases such as hay fever, extrinsic asthma, drug hypersensitivities and some forms of urticaria are mediated by allergen-specific antibodies of the IgE class, known also as reagins. Specific suppression of the IgE antibodies to haptens and protein antigens has been achieved recently by injection of conjugates of haptens with non-immunogenic carriers¹⁻⁵ or by appropriately modified allergens⁶⁻⁸. Here we describe a new method for converting a well defined protein antigen, such as ovalbumin (OA), or the mixture of the non-dialysable allergenic constituents of the aqueous extract of ragweed pollen (RAG), to tolerogenic and non-allergenic products which are capable of abrogating the primary as well as ongoing IgE responses to the corresponding native allergens in B₆D₂F₁ mice and rats. Our method involves the covalent coupling of the allergens to the non-immunogenic, hydrophilic polymer, polyethylene glycol (PEG); this approach was inspired by the report that coupling of PEG to catalase and bovine serum albumin resulted in loss of the immunogenicity of these proteins⁹.

In the present study two batches of PEG (Fisher Scientific Company, New Jersey, USA) with average molecular weights of 6,000 and 20,000, referred to as PEG₆ and PEG₂₀, were coupled to OA and RAG using cyanuric chloride as the coupling reagent¹⁰. After a number of trials, it was shown that PEG-antigen conjugates, possessing the required tolerogenic capacity, were prepared by adding cyanuric chloride (0.2 g in 3 ml of *N,N'*-dimethylformamide) to a mixture of 0.4 g of PEG dissolved in 6 ml of 0.5 N NaOH and 0.1 g of OA or RAG in 5 ml of phosphate-buffered saline, pH 7.2. These conjugates were separated from the original reactants by filtration through Sepharose 4B. The chemical characteristics of these conjugates were not studied in detail in these exploratory investigations and chemically well defined PEG-antigen conjugates, differing in the number and molecular weight of PEG molecules grafted on to each antigen molecule, are currently being synthesised.

Optimal anti-DNP and anti-OA IgE responses were induced in B₆D₂F₁ mice and in Chester Beatty rats and IgE responses specific for RAG, antigen E of RAG and the antigens of the extract of *Ascaris suum* (Asc) were induced in B₆D₂F₁ mice by procedures similar to those described previously^{4,11}. Serum IgE levels were determined by passive

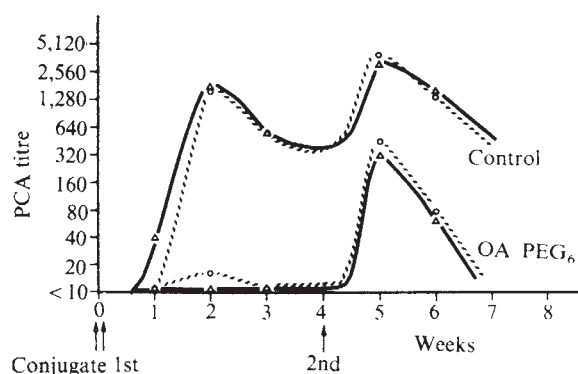


Fig. 1 Suppression of the reagenic antibody response with OA-PEG₆. Test mice were injected i.v. with 1 mg of OA-PEG₆ 4 h before receiving the first sensitising dose of DNP-OA on day 0. The mice received i.p. a second sensitising dose of antigen on day 28 without further administration of the conjugate. Δ — Δ , Anti-OA, IgE response; $\circ$ — $\circ$, Anti-DNP IgE response.

cutaneous anaphylaxis (PCA) assays in random-bred hooded rats⁴.

Intravenous (i.v.) administration of 1 mg of OA-PEG₆ into mice 4 h before immunisation with 1 μ g of DNP₃-OA in Al(OH)₃, referred to as the sensitising dose of DNP-OA, suppressed completely the primary anti-DNP and anti-OA IgE responses and these mice responded very poorly to a further sensitising dose of DNP-OA (Fig. 1). By contrast, injection of 1 mg of free PEG₆ or PEG₂₀, or of OA which had been treated with cyanuric chloride in absence of PEG, had no effect on the ability of the animals to mount IgE responses to DNP-OA. The specificity of the immunosuppression was demonstrated by the results listed in Table 1. Thus, whereas treatment of mice with OA-PEG₆ suppressed profoundly their ability to produce IgE antibodies to both DNP and OA on sensitisation with DNP-OA, it did not affect their capacity to produce IgE antibodies to both DNP and Asc on immunisation with DNP-Asc. Hence, the mechanism of immunological suppression induced by OA-PEG seems to operate at the level of cells interfering with the normal cooperation between helper T cells and B₆ cells¹².

In relation to the potential use of PEG-allergen conjugates for the immunotherapy of allergic individuals, it is to be noted that i.v. injection of OA-PEG conjugates into mice presensitised with DNP-OA led to the suppression of their ongoing anti-DNP and anti-OA reagenic responses.

Table 1 Specificity of immunosuppression with OA-PEG

Group	Treatment		Day	Primary immunisation			PCA Titres [§]			
	Day 0	Day 28		Anti-DNP	Anti-OA	Anti-Asc	Day	Anti-DNP	Anti-OA	Anti-Asc
Control A*	DNP-OA	DNP-OA	14	1,000	1,000	NT	35	3,310	3,500	NT
							42	1,280	3,020	NT
Test A†	OA-PEG ₆ plus DNP-OA	DNP-OA	14	< 10	< 10	NT	35	80	480	NT
							42	480	860	NT
Control B*	DNP-Asc	DNP-Asc	14	890	NT	200	35	1,000	NT	1,500
							42	640	NT	840
Test B‡	OA-PEG ₆ plus DNP-Asc	DNP-Asc	14	600	NT	180	35	1,650	NT	1,700
							42	780	NT	640

*Mice in control groups A and B received on each of days 0 and 28 a sensitising dose of DNP-OA or 10 μ g of DNP Asc in Al(OH)₃, respectively.

†Mice in test group A received an i.v. injection of 800 μ g OA-PEG₆ 4 h before administration of the sensitising dose of DNP-OA on day 0 and were challenged with the same dose of DNP-OA on day 28.

‡Mice in test group B received an i.v. injection of 800 μ g OA-PEG₆ 4 h before sensitisation with 10 μ g DNP-Asc in Al(OH)₃ on day 0 and were challenged with the same dose of DNP-Asc on day 28.

§Groups of four to five mice were bled on day 14 for the determination of the primary IgE response and on days 35 and 42 for assessing the level of IgE antibodies in the secondary response; the sera of each group were pooled prior to PCA testing (NT, not tested).

Table 2 Induction of tolerance in rats with OA-PEG₂₀

Group	Treatment*	Rat no.	PCA Titres†		Systemic Reaction‡
			Anti-DNP	Anti-OA	
Control	DNP-OA + Al(OH) ₃ + 10 ¹⁰ <i>B. pertussis</i>	1	90	80	Death in 24 min
		2	260	100	Death in 33 min
		3	260	100	Death in 36 min
		4	270	80	Death in 28 min
Test	OA-PEG ₂₀ and DNP-OA + Al(OH) ₃ + 10 ¹⁰ <i>B. pertussis</i>	5	< 10	< 10	No symptoms
		6	20	20	No symptoms
		7	< 10	< 10	No symptoms
		8	< 10	< 10	No symptoms

*All rats were sensitised i.p. with 1 µg DNP₃-OA in presence of 1 mg Al(OH)₃ and 10¹⁰ *Bordetella pertussis*; the rats in the test group received an additional i.v. injection of 2.5 mg of OA-PEG₂₀ 4 h before sensitisation.

†The PCA titres refer to the reaginic antibodies in sera of rats 14 d after sensitisation.

‡Rats were challenged i.v. with 2 mg of OA in 1 ml PBS 14 d after sensitisation.

Similarly, the administration of either RAG-PEG₆ or RAG-PEG₂₀ into mice, which had been sensitised with RAG, abrogated their ongoing anti-RAG IgE response and further sensitisation with RAG did not restore their anti-RAG IgE antibody levels (Fig. 2). The results given in Table 2 indicate that tolerance was also induced in rats by i.v. injection of OA-PEG₂₀ 4 h before sensitisation with DNP-OA and, most significantly, whereas rats made tolerant by this treatment did not show any discomfort on i.v. challenge with OA, the control-sensitised rats died of anaphylaxis after receiving OA.

In addition to being tolerogenic, PEG-allergen conjugates were shown to be devoid of the ability to react with antibodies to the unmodified allergens. Thus, these conjugates failed to combine with antibodies to the native allergens, as proven by their inability (1) to neutralise *in vitro* IgE antibodies to the unmodified allergens (demonstrated by PCA as described in ref. 13), (2) to give precipitin bands with rabbit antisera specific for the native allergens, (3) to induce anaphylaxis in sensitised rats and (4) to trigger PCA reactions in skin sites sensitised with IgE antibodies to the native allergens. Moreover, it is noted that the lack of allergenicity of RAG-PEG₂₀, was also confirmed with respect to human reagins to RAG. Thus, in experiments involving the direct skin testing of a RAG-sensitive individual and PCA testing of a rhesus monkey sensitised with a reaginic serum of a patient allergic to RAG, RAG-PEG₂₀ was shown to be essentially devoid of the allergenicity of native RAG. Hence, PEG conjugates of common allergens may prove to be useful therapeutic agents for the abrogation of IgE-mediated allergies without unleashing anaphylactic reactions. The lack of allergenicity and antigenicity of these tolerogenic PEG conjugates was also paralleled by their non-immunogenicity, that is, their

inability to induce an antibody response in normal mice in absence of adjuvants.

After induction of tolerance with PEG-antigen conjugates in mice, the state of unresponsiveness could be maintained even after transfer of tolerised spleen cells into X-irradiated recipients, that is, challenge of the recipients with a sensitising dose of the antigen failed to elicit a secondary response. Furthermore, the results of more recent experiments indicate that the suppressive effects due to PEG-modified antigens were caused by suppressor cells specific for the determinants of the carrier portion of the native antigens. This observation is in agreement with the recent demonstration⁷ that urea-denatured antigen E of RAG also generated suppressor T cells and one may, therefore, suggest that an antigen in a non-immunogenic form favours the proliferation of T suppressor cells. The cellular mechanism underlying the observed suppression of the IgE response by PEG-antigen conjugates remains to be elucidated with chemically better defined PEG-antigen conjugates. We expect that the number and molecular weight of PEG molecules grafted on to each antigen molecule would affect the structure of the determinants of the antigen as well as their accessibility to the free and cell membrane-bound immunoglobulins and to receptors on helper, suppressor and accessory cells involved in the overall immune response.

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Note added in proof: It has been recently reported¹⁴ that coupling of antigen E of RAG to PEG of molecular weight 2,100 resulted in pronounced reduction of the allergenicity and immunogenicity of this antigen. No indication was given if this conjugate was immunosuppressive, which is the most prominent feature of the conjugates prepared by us.

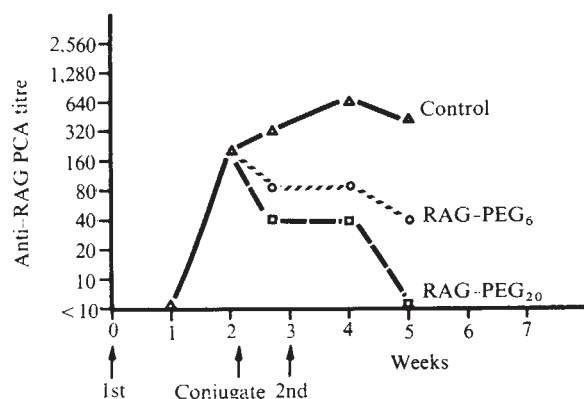
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- Katz, D. H., Hamaoka, T. & Benacerraf, B. *J. exp. Med.* **136**, 1404-1429 (1972).
- Havas, H. F. *Immunology* **17**, 819-829 (1969).
- Golan, D. T. & Borel, Y. *J. exp. Med.* **134**, 1046-1061 (1971).
- Lee, W. Y. & Sehon, A. H. *J. Immun.* **114**, 829-836 (1975).
- Lee, W. Y. & Sehon, A. H. *J. Immun.* **117**, 927-934 (1976).
- Ishizaka, K., Okudaira, H. & King, T. P. *J. Immun.* **114**, 110-115 (1975).
- Takatsu, K. & Ishizaka, K. *J. Immun.* **116**, 1257-1264 (1976).
- Bach, M. K. & Brashler, J. R. *J. Immun.* **114**, 1799-1807 (1975).
- Abuchowski, A., thesis, Rutgers Univ., New Brunswick, N.J. (1975).
- Sharon, R., McMaster, P. R. B., Kask, A. M., Owens, J. D. & Paul, W. E. *J. Immun.* **114**, 1585-1589 (1975).
- Pan, D., Lee, W. Y., Sehon, A. H. & Bazin, H. *Fedn Proc.* **35**, 433 (1976).
- Green, I., Paul, W. E. & Benacerraf, B. *J. exp. Med.* **127**, 43-53 (1968).
- Lee, W. Y. & Sehon, A. H. *J. Immun.* **114**, 837-842 (1975).
- King, T. P., Kochoomian, L. & Lichtenstein, L. M. *Archs Biochem. Biophys.* **178**, 442-450 (1977).

Fig. 2 Abrogation of the reaginic antibody response to ragweed allergens (RAG). Test mice were immunised i.p. with 10 µg of RAG in presence of Al(OH)₃ on day 0 and 800 µg of either RAG-PEG₆ or RAG-PEG₂₀ were injected i.v. on day 15. The mice received i.p. a second sensitising dose of the antigen on day 21 without further administration of the conjugate.



Retinyl acetate inhibits mammary carcinogenesis induced by *N*-methyl-*N*-nitrosourea

THE use of pharmacological agents to prevent development of epithelial cancer is attracting increasing attention. Recent studies have shown the successful use of vitamin A and its synthetic analogues (together known as retinoids) for this purpose^{1,2}. We have shown that both natural and synthetic retinoids (retinyl acetate and retinyl methyl ether, respectively) markedly inhibit mammary carcinogenesis induced in the Sprague-Dawley rat by 7,12-dimethylbenz[*a*]anthracene (DMBA) (refs 3, 4). Although the DMBA model has contributed significantly to the understanding of mammary carcinogenesis, particularly hormone responsiveness, the model is less than ideal in that the tumours only rarely invade surrounding tissues and do not metastasise. A new mammary carcinogenesis model, first described by Gullino *et al.*⁵, using intravenous injection of *N*-methyl-*N*-nitrosourea (NMU) in the Buffalo rat, satisfies the deficiencies of the DMBA model in that cancers are exclusively localised in the mammary gland, are hormone dependent, show metastasis to other organs, are transplantable, and result in hypercalcaemia. NMU also causes a high incidence of mammary cancers in female Sprague-Dawley and Fischer-344 rats⁶, but the biological characteristics of the cancers in these strains have not been described in detail. But, our preliminary studies using Sprague-Dawley rats indicated that NMU-induced mammary cancers are highly aggressive and similar histologically to those induced in the Buffalo rat. The NMU model of mammary cancer thus resembles the human disease more closely than the DMBA model. We describe here a study of the effect of a retinyl acetate on mammary carcinogenesis induced by NMU, using a modification of the procedure reported by Gullino *et al.*⁵. We show that retinyl acetate inhibits carcinogenesis in this system.

Female rats of the Sprague-Dawley strain were obtained when 42 days of age. All the animals initially received stock diet (Wayne Lab Meal) and tap water *ad libitum*. NMU was dissolved in physiological saline (20 mg ml⁻¹) which had been adjusted to pH 5.0 with a few drops of 3% acetic acid. Rats (50-d-old: average weight 150 g) were injected intravenously with NMU at 5.0, 2.5, or 1.25 mg per 100 g of body weight (Table 1). Control animals received saline alone. Each NMU-treated and control rat received a second injection 7 days later. Three days after the second injection, all rats were placed on the diets indicated in Table 1 for the remainder of the experiment. Retinyl acetate was blended into the stock diet in the form of stable gelatinised beadlets at a concentration of 250 mg

retinyl acetate per kg diet; rats in groups 1, 3, 5, and 7 received the control gelatinised beadlet material without retinoid (placebo beadlets). All rats were palpated twice each week for detection of mammary tumours, and were also weighed twice each week and observed daily for evidence of retinoid toxicity. At 175 days all animals were sacrificed and the mammary tumours removed and processed for histological classification. More than 75% of the tumours (Table 1) were adenocarcinomas, which were highly aggressive, contained many mitotic figures, and were invasive to the pectoral muscle. Metastasis to the kidney was found in one rat in group 5. A few benign tumours were also seen, and these were either adenomas or fibroadenomas.

No mammary tumours developed in animals which received no carcinogen. Animals receiving the high dose of NMU and fed placebo diet (group 3) had a mammary cancer incidence of 83% (Table 1), while animals treated with this dose of carcinogen and fed retinyl acetate had a mammary cancer incidence of only 50% (group 4). A similar reduction in the incidence of mammary cancer was found in rats receiving retinyl acetate and the intermediate dose of NMU (group 6 compared with group 5). No mammary cancer developed in those rats receiving the low dose of NMU and retinyl acetate (group 8), while the animals treated with this dose of NMU and placebo diet (group 7), had a 20% incidence of cancer. Although this incidence of mammary cancer in group 7 might be considered relatively low, it is more than twice that found in the total population of women at risk in England or the United States⁶.

In addition to reducing the incidence of mammary cancer, retinyl acetate also caused a marked reduction in the total number of cancers, as well as benign tumours (Table 1). Furthermore, the administration of retinyl acetate greatly prolonged the latency period of mammary cancer at all three doses of NMU. As shown in Fig. 1, the time of appearance of the first palpable cancer in rats treated with the high dose of NMU was doubled by the administration of retinyl acetate. With the low dose of carcinogen, the first palpable cancer appeared at 107 days, whereas animals fed retinyl acetate after the low dose of NMU were free of mammary cancer on termination of the experiment at 175 days.

The mechanism by which retinoids, both natural and synthetic, protect against mammary carcinogenesis is unknown. Here, cancer prevention was not due to an inhibition of initiation of cancer, since feeding of retinyl acetate was not begun until after all free carcinogen had disappeared. The half life of NMU at body pH is only a few hours⁷. Moreover, inhibition of carcinogenesis did not result from some general toxic effect of retinyl acetate, as 'hypervitaminosis A' (ref. 8) was not observed. At necropsy,

Table 1 Effect of retinyl acetate on mammary carcinogenesis

Group	NMU	Retinoid	No. of rats with cancer	Incidence of mammary cancer (%)	Total no. of tumours	
			Total no. of rats		Cancer	Benign
1	None	Placebo	0/30	0	0	0
2	None	Retinyl acetate	0/30	0	0	0
3	High dose	Placebo	25/30	83	61	17
4	High dose	Retinyl acetate	15/30	50*	25*	4*
5	Intermediate dose	Placebo	12/29	41	16	3
6	Intermediate dose	Retinyl acetate	7/30	23*	11*	1
7	Low dose	Placebo	6/30	20	7	2
8	Low dose	Retinyl acetate	0/30	0*	0*	0

Female Sprague-Dawley rats received either saline or NMU in saline (5.0, 2.5, or 1.25 mg per 100 g body weight) by i.v. injection at 50 d of age. A second injection was administered one week later. The animals were placed on either retinyl acetate (250 mg per kg of diet) or placebo beadlets 3 d after the second injection and sacrificed 175 d after the initial NMU injection.

*Significantly different from respective placebo control group, $P < 0.05$ or better.

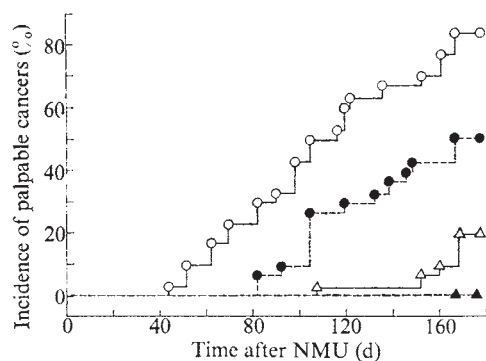


Fig. 1 Effect of retinyl acetate on the time of appearance of palpable mammary cancers which were confirmed histologically at necropsy. Rats were placed on retinyl acetate or placebo diet 3 d after the second i.v. injection of either 5.0 mg or 1.25 mg NMU per 100 g body weight. The rats were palpated for mammary tumours twice weekly for the duration of the experiment. Treatments were: ○, high dose NMU, placebo diet; ●, high dose NMU, retinyl acetate diet; △, low dose NMU, placebo diet; ▲, low dose NMU, retinyl acetate diet.

body weight and liver histology of rats fed retinyl acetate were essentially identical to those fed placebo diet. Vaginal smears from rats fed retinyl acetate revealed normal oestrous cycles, suggesting that metabolism of ovarian steroids was not significantly altered. Although the fundamental effects of retinoids in maintenance of normal epithelial cell differentiation (even in tissues exposed to carcinogens *in vitro*^{9,10}) suggest that this process may be the mechanism of inhibition of carcinogenesis^{1,2}, other effects on the endocrine or immune system cannot be ruled out at the present time.

The present data demonstrate the efficacy of retinyl acetate in prevention of experimental mammary cancer. Although retinoid toxicity was not seen in these studies, the potential toxicity of retinyl esters, such as retinyl acetate or retinyl palmitate^{8,11}, makes the development of new synthetic retinoids, with greater activity and lesser toxicity, of significant importance.

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- Sporn, M. B., Dunlop, N. M., Newton, D. L. & Smith, J. M. *Fedn Proc.* 35, 1332-1338 (1976).
- Sporn, M. B. *Nutr. Rev.* 35, 65-69 (1977).
- Moon, R. C., Grubbs, C. J. & Sporn, M. B. *Cancer Res.* 36, 2626-2630 (1976).
- Grubbs, C. J., Moon, R. C., Sporn, M. B. & Newton, D. L. *Cancer Res.* 37, 599-602 (1977).
- Gullino, P. M., Pettigrew, H. M. & Grantham, F. H. *J. natn. Cancer Inst.* 54, 401-414 (1975).
- Doll, R. *Nature* 265, 589-596 (1977).
- Druckrey, H., Preussmann, R., Ivankovic, S. & Schmähel, D. *Z. Krebsforschung* 69, 103-201 (1967).
- Moore, T. in *The Vitamins* 2nd edn (eds Seibell, W. H. & Harris, R. S.) 1, 280-294 (Academic, New York, 1967).
- Lasnitzki, I. *Br. J. Cancer* 9, 434-441 (1955).
- Chopra, D. P. & Wilkoff, L. J. *J. Natn. Cancer Inst.* 56, 583-589 (1976).
- Smith, F. R. & Goodman, D. S. *New Engl. J. Med.* 294, 805-808 (1976).

Synthetic polyamines added to cultures containing bovine sera reversibly inhibit *in vitro* parameters of immunity

POLYAMINES are present in high concentration in foetal¹ and neoplastic tissue² and seminal fluid³—all representing antigenic challenges which usually fail to elicit the appropriate immune response. This has prompted us to investigate the influence of polyamines on *in vitro* parameters of immunity. We report here that micromolar quantities of spermine and spermidine reversibly inhibit three responses of primary cultures of murine spleen cells. These are (1) DNA synthetic response stimulated by phytohaemagglutinin (PHA), pokeweed mitogen (PWM), concanavalin A (con A) or bacterial lipopolysaccharide (LPS); (2) mixed lymphocyte response (MLC) stimulated by irradiated allogeneic cells; and (3) the induction but not the expression of the cytolytic lymphocyte response. The inhibition does not seem to be due to toxicity but occurs only in the presence of calf or foetal calf sera.

When micromolar quantities of spermine and spermidine

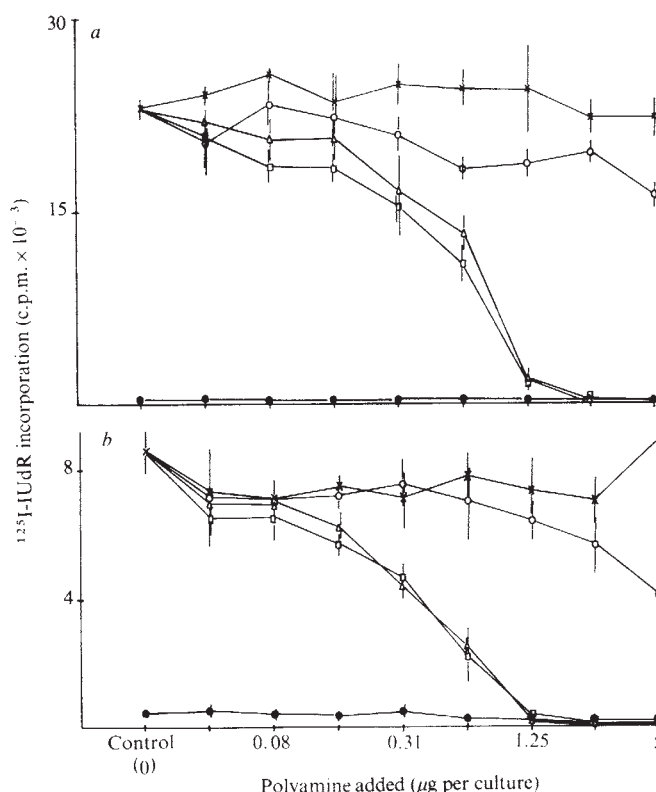


Fig. 1 Effect of polyamines on lymphocyte mitogen responses. 5×10^5 BALB/c spleen cells were cultured in flat bottom Falcon microtest II plates in a volume of 200 μ l in RPMI 1640 medium with 5% FCS which contained nil or a, 0.25 μ g con A (Pharmacia) or b, 1.0 μ g *E. coli* 055:B5 lipopolysaccharide (Difco). The indicated amount of polyamine (obtained from Calbiochem as cadaverine dihydrochloride, putrescine dihydrochloride, spermidine trihydrochloride and spermine tetrahydrochloride dissolved in serum-free medium) was added in 50 μ l making the final volume 250 μ l. Cultures were pulsed with 0.1 μ Ci 125 I-IUDR (250 μ Ci μ g⁻¹) (New England Nuclear) after 44 h of culture and cells were collected 18 h later using a multiple automated sample collector (Otto Hiller, Madison, Wisconsin). Samples were counted in a Packard automatic gamma spectrometer. The mean and standard deviations of quadruplicate cultures are shown. Polyamines tested were putrescine ($\text{NH}_2(\text{CH}_2)_4\text{NH}_2$) (×), cadaverine ($\text{NH}_2(\text{CH}_2)_6\text{NH}_2$) (○), spermine ($\text{NH}_2(\text{CH}_2)_3\text{NH}(\text{CH}_2)_4\text{NH}(\text{CH}_2)_3\text{NH}_2$) (□) and spermidine ($\text{NH}_2(\text{CH}_2)_3\text{NH}(\text{CH}_2)_4\text{NH}_2$) (△). All cultures which did not contain mitogen (●) but did contain varying concentrations of polyamine gave essentially the same low incorporation rate as shown by this representative plot.

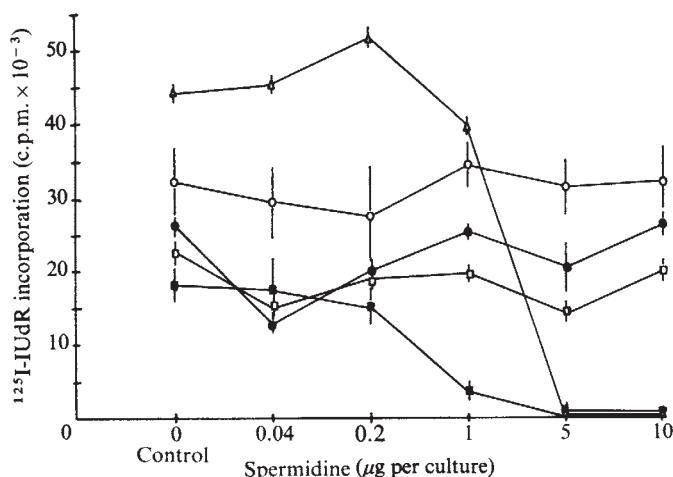


Fig. 2 Serum requirements for spermidine inhibition of mitogen responsiveness. Spleen cells were cultured, additions made, and DNA synthesis measured as described in the legend to Fig. 1, except that the amount of mitogen per culture was 0.5 μ g con A. FCS (▲), calf serum (■) obtained from calves 3–6 months of age, and human serum (○) were present at a final concentration of 4% and mouse serum (●) at 0.4%. Cells cultured in serum-free medium are represented by (□). Essentially identical curves were obtained for PHA, PWM and LPS.

were added to cultured murine spleen cells stimulated with LPS or con A, the DNA synthetic response was inhibited. Identical doses of putrescine and cadaverine had no effect (Fig. 1). Similar results were obtained for PWM and PHA stimulated cultures. Larger doses of polyamine were required to suppress the response to PHA or con A than to suppress that to LPS and PWM. The inhibition could be demonstrated with either calf or foetal calf serum (FCS) in the cultures. In parallel cultures with human or mouse serum, or with no serum, there was no inhibition in spite of the presence of up to 10 μ g per well of spermine and spermidine (Fig. 2).

When FCS was titrated into cultures containing a constant 4% human serum, polyamines inhibited LPS and con A stimulated mitogenesis when the FCS concentration reached 0.5–1.0%. This suggests that some component of FCS is essential for the inhibition.

We next tested whether the observed inhibition was due to toxicity. First we examined the effect of spermine added at different times after inception of standard cultures

stimulated with either con A or LPS. When spermine was added up to 29 h after inception of culture, it could completely inhibit the LPS response. To inhibit the con A completely, spermine had to be added within 22 h of culture inception. The later the polyamine was added, the more was required to obtain the same level of inhibition. This argues against a simple mechanism of acute toxicity but is consistent with an effect on some early event in the differentiation of mitogen-stimulated lymphoid cells.

To investigate the reversibility of the inhibition, we preincubated murine spleen cells (2.5×10^7 in 50 ml of medium RPMI 1640 and a final concentration of 2% FCS with no additions or with spermine ($10 \mu\text{g ml}^{-1}$) or spermidine ($10 \mu\text{g ml}^{-1}$) for either 24, 48 or 72 h. Cultures were then collected, washed three times and subcultured in the microtest II system (see legend of Fig. 1) where they were stimulated with PHA, PWM, con A, or LPS. The responses were compared with those of fresh murine spleen cells obtained each day and cultured in parallel. Control cells, preincubated for 24 h without polyamine, responded well (although less than did fresh cells) to each mitogen when tested in subculture: cells preincubated for 24 h with supra-inhibitory doses of polyamine responded as well as did preincubated control cells in culture.

Cells cultured with and without polyamine for 48 h responded less well to T cell mitogens in general, but there was no significant difference between those preincubated without polyamine and those preincubated in spermidine. By this time, however, all preincubated cells have lost their ability to respond to LPS and PWM and the level of radiolabel incorporation by preincubated cells subcultured without stimulus has increased significantly.

After 3 d of preincubation no cells responded to the mitogens when subcultured. There was no significant difference in the recovery of total viable cells preincubated in spermidine compared to control medium.

The fact that spermine or spermidine at 5–10 μ g per well completely inhibited the response to mitogen when added at the inception of culture (Fig. 1) or 22 h later, but not after preincubation in either polyamine for 24 h suggests that a toxic principle was not responsible for the lack of iododeoxyuridine (IUdR) incorporation in the subcultures. It is consistent with polyamine affecting some event in stimulated cells rather than having a nonspecific deleterious effect on quiescent cells.

Polyamines also inhibit the ability of murine lymphocytes to recognise irradiated allogeneic cells (Table 1). Furthermore, the ability of cells in a mixed lymphocyte culture to

Table 1 Influence of synthetic polyamines on murine mixed lymphocyte and cell-mediated cytotoxicity responses of CBA spleen cells stimulated by irradiated DBA/2 spleen cells

	Spermine added (μ g)	^{125}I -IUdR incorporated (c.p.m.) $\times 10^3$		
		Day 4	Day 5	Day 6
5×10^5 CBA + 5×10^5 DBA	nil	26.0 $\pm$ 5.00	64.6 $\pm$ 2.70	30.9 $\pm$ 3.06
	0.31	15.4 $\pm$ 3.40	40.5 $\pm$ 9.19	32.7 $\pm$ 6.14
	1.25	0.1 $\pm$ 0.02	0.1 $\pm$ 0.02	0.1 $\pm$ 0.12
	5	0.1 $\pm$ 0.01	0.1 $\pm$ 0.02	0.1 $\pm$ 0.02
	20	0.1 $\pm$ 0.04	0.3 $\pm$ 0.13	0.5 $\pm$ 0.06
5×10^5 DBA + nil	nil	0.3 $\pm$ 0.12	1.6 $\pm$ 1.7	0.9 $\pm$ 0.54
			% Chromium released	
	nil		56.3	
	0.31		59.1	
	1.25		7.2	
	5.0		1.8	
	20.0		0	

^{125}I -IUdR incorporation data are means of four replicate cultures $\pm$ 1 s.d. Cells were collected from identical prepared cultures on days 4–6 with the peak control response occurring on day 5. Note that as little as 310 ng of spermine significantly suppressed the response while 1.2 μ g or more of spermine completely suppressed the response. Where polyamine was present only during the expression phase of the cytotoxic response the percentage of chromium release was in excess of 45%. Addition of as much as 20 μ g of polyamine to chromated P-815 target cells alone showed only a 1–2% release of chromium indicating that polyamine *per se* is not acutely toxic to cells known for their fragility.

mount a non-complement-dependent cytolytic response is impaired if polyamines are present from the inception of the culture. Polyamines present only during the chromium release phase of culture do not alter the response nor do they increase the spontaneous release of chromium from the target cells.

These results indicate that certain polyamines have a reversible, suppressive effect on *in vitro* parameters of immunity; a finding of interest for several reasons. The inhibitory property of the product of bovine serum-polyamine interaction may explain why there is only limited DNA synthesis or antibody formation in most studies of *in vitro* immune responses. Lymphocytes contain nanomolar quantities of spermine⁴ and in culture they are normally supplemented with bovine serum. In most *in vitro* systems 60–80% of cells die and probably sufficient polyamine could be released into the medium to react with the bovine serum, form the inhibitor and thus render the response self-limiting.

Spermine and spermidine themselves are not inhibitory; they must either be converted into some other active moiety by the bovine serum or alternatively cause the bovine serum to release an unrelated active molecule.

The possibility that the observations described here were due to toxicity was suggested by a report that a specific amine oxidase of ruminant sera can convert spermine or spermidine to aminoaldehydes toxic to cell cultures⁵. Also, Alarcon found acrolein ($\text{CH}_2\text{CH:CHO}$) in mixtures of bovine sera and polyamine, and wondered whether this might account for the toxicity of the mixtures because acrolein is toxic⁶. We do not believe, however, that either possibility applies to our observations. The strongest evidence against toxicity is the finding that cells preincubated for 24–48 h in supra-inhibitory doses of spermidine later responded to mitogen stimulation as well as did cells preincubated in control medium. It seems unlikely that fixatives such as acrolein or aminoaldehyde would reversibly fix the lymphocyte surface.

Cells involved in the immune response seem to be particularly susceptible to polyamines compared with fibroblasts, for example. Polyamines are widely distributed in mammalian tissues⁷. They are also present in very high concentration in foetal and neoplastic tissues; both of which are known to possess specific antigens but still avoid immunological rejection^{1,8,9}. We postulate that the successful subversion of the host immune system in those individuals bearing tumours or outbred fetuses is mediated by polyamine or a polyamine interaction product. It is conceivable that the product of the interaction of polyamine and bovine serum could inhibit immune reactivity in a general way, and thus represent a natural immunoregulatory agent.

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¹ Janne, J., Raina, A. & Siimes, M. *Acta physiol. Scand.* **62**, 352–358 (1964).

² Russell, D. H. & Snyder, S. H. *Proc. Natn. Acad. Sci. U.S.A.* **60**, 1420–1427 (1968).

³ Williams-Ashman, H. G. & Lockwood, D. H. *Ann. N.Y. Acad. Sci.* **171**, 882–894 (1970).

⁴ Fillingame, R. H. & Morris, D. R. in *Polyamines in Normal and Neoplastic Growth*, 249 (Raven, New York, 1973).

⁵ Tabor, C. W., Tabor, H. & Bachrach, U. *J. biol. Chem.* **239**, 2194–2203 (1964).

⁶ Alarcon, R. A. *Archs Biochem. Biophys.* **137**, 365–372 (1970).

⁷ Rosenthal, S. M. & Tabor, C. W. *J. Pharmac. exp. Ther.* **116**, 131–138 (1956).

⁸ Russell, D. H. *Nature* **233**, 144 (1971).

⁹ Snyder, S. H. & Russell, D. H. *Fedn Proc.* **29**, 1575 (1970).

Identification of a thymic inhibitor ('chalone') of lymphocyte transformation as a spermine complex

CHALONES are defined as endogenous, tissue-specific but species nonspecific inhibitors of cell proliferation. Their potential use in the control of neoplasia is well recognised^{1–3}. Circumstantial evidence for the existence of a lymphocyte chalone has been produced^{4–8}, but neither its chemical nature nor its molecular weight have been satisfactorily ascertained. During our search for a lymphocyte chalone, an inhibitor of lymphocyte transformation has been reproducibly isolated from porcine thymic tissue. We report here that though the inhibitor was non-dialysable, its active moiety was identified biologically and chemically as spermine, molecular weight only 202. This accords with the formation in thymic extracts of a tightly bound spermine complex. As the complex displayed some tissue specificity it is possible that an unidentified tissue-specific material acts as a carrier for spermine. In relatively crude extracts of tissues it is probable that spermine-elicited inhibition swamps any authentic chalone activity.

Extracts of thymic tissue were prepared and fractionated by the method of Kiger *et al.*⁵. Briefly, ethanol was added (4 °C) to an aqueous thymic extract (fraction TAE) to a concentration of 42% by vol. The soluble portion (fraction T₄) was dialysed (Visking tubing) against distilled water and subjected to ion-exchange separation on DEAE A-50 Sephadex (Pharmacia). A fraction analogous to (F₂–F₆) of Kiger *et al.*⁵ was obtained, and thoroughly dialysed against distilled water. Fractions were freeze-dried for storage at –20 °C. The most highly purified (F₂–F₆) sub-fraction contained a mixture of material with a molecular weight ranging from 50,000 down to 800 as defined by P-60 and P-2 (Biorad) gel filtration.

Table 1 Comparison of inhibitory doses (ID) of thymic extracts and polyamines on PHA-stimulated lymphocyte, fibroblast and granulocyte uptake of ³HTdR (DNA synthesis) in medium containing foetal calf serum

Fraction	Lymphocytes ID ₅₀	Fibroblasts ID ₅₀	Granulocytes ID ₅₀
TAE	275 µg ml ⁻¹	>1,000 µg ml ⁻¹	>1,000 µg ml ⁻¹
T ₄	210 µg ml ⁻¹	1,000 µg ml ⁻¹	1,000 µg ml ⁻¹
(F ₂ –F ₆)	100 µg ml ⁻¹	500 µg ml ⁻¹	500 µg ml ⁻¹
(F ₂ –F ₆) polyamines	20 µg ml ⁻¹	20 µg ml ⁻¹	25 µg ml ⁻¹
Spermine	12 µM	12 µM	15 µM
Spermidine	36 µM	36 µM	n.t.
Putrescine	>160 µM	n.t.	n.t.

Triplicate 1.0 ml cultures of 5 × 10⁶ young male rat (Sprague-Dawley strain) thymic lymphocytes were incubated with 15 µg of purified PHA (Wellcome) in 75 × 10 mm tubes (Falcon) for 62 h, and uptake from 1.0 µCi of ³HTdR (2 Ci mmol⁻¹) or ³HUdR (10.3 Ci mmol⁻¹, Radiochemical Centre) was measured for the last 16 h of incubation. Hamster lung fibroblasts (Yerganian cell line) were established in Leighton tubes (1.0 ml triplicate cultures) and uptake of 1.0 µCi was measured over one generation of cells (16 h). Rat granulocytes were assayed by the method of Paukovits¹⁷. Medium RPMI 1640 (Flow) containing antibiotics, 2 mM L-glutamine, 16 mM NaHCO₃ (buffer) and 12.5% foetal calf serum (Rehatuin, Armour) were used for all cultures. The same batch of serum was used throughout. Completed media containing thymic extracts or polyamine were incubated for 1–2 h before adding cells. Reagents were sterilised by filtration through Millipore 0.22 µm membranes. Cultures were incubated at 37 °C in an atmosphere of 5% CO₂ in air. Results were calculated from a dose-response curve plotted for the mean d.p.m. (background subtracted) of triplicate cultures. Standard deviations were less than 12% of the mean. Relative uptake of ³HUdR was similar to that for ³HTdR and so is not considered in the Table. Measurement of incorporation of radio labels in TCA-precipitable material of washed collected cells in PCS scintillator (Searle) was made using a Beckman spectrophotometer. n.t., Not tested.

Thymic fractions TAE, T₄ and (F₂-F₆) inhibited DNA synthesis (measured by uptake of ³HTdR) and RNA synthesis (uptake of ³HUdR) equally, in phytohaemagglutinin (PHA)-stimulated rat thymic lymphocytes. In contrast, the inhibition was significantly less when assayed against fibroblast or granulocyte proliferation. Representative results are summarised in Table 1. In addition, administration of fraction (F₂-F₆) to CBA recipient mice for full-thickness WH strain mouse tail-skin allografts at a dosage of 7.0 mg per mouse on days -1, 1, 3 and 6 after grafting, produced significant ($P=4.75$ for 12 d.f.) but not dramatic prolongation of graft survival from 10.14 ± 0.28 (s.d.) to 12.86 ± 0.51 d.

Thus the extract (F₂-F₆) was shown to exhibit chalone-like activity both *in vitro* and *in vivo*. A remarkable finding was that all the above fractions completely lost inhibitory activity when instead of foetal calf serum, human serum (adult or foetal-cord) was used to supplement cultures. Results using fraction (F₂-F₆) are shown in Fig. 1. Byrd *et al.*⁹ have reported that potent inhibition of lymphocyte transformation was evoked by the polyamines spermine and spermidine, and that the phenomenon was similarly dependent on the presence of bovine serum. We have observed similar effects and shown fibroblasts and granulocytes to be equally susceptible (Table 1); putrescine was not an inhibitor. Moreover, injection of 1.0-mg doses of spermidine (Sigma) in the schedule described above caused a somewhat longer retention of skin grafts than previously obtained with fraction (F₂-F₆).

Experiments still in progress indicate that the inhibitory properties of the polyamines are closely linked to plasma amine oxidase activity, which is abundant in ruminant sera but shows little activity in human sera. If this is so, the aldehydes produced by the enzymic oxidative deamination of spermine and spermidine must be exceedingly potent inhibitors. Consequently, our investigations were continued to explore the possibility of the thymic fractions containing

Fig. 1 Inhibitory activity of thymic fraction (F₂-F₆) on the uptake of ³HTdR by PHA-transformed lymphocytes in foetal calf serum (●) and absence of activity in human serum (○). Culture details are presented in Table 1. Each point represents the mean d.p.m. of triplicate cultures $\pm$ s.d. (background subtracted).

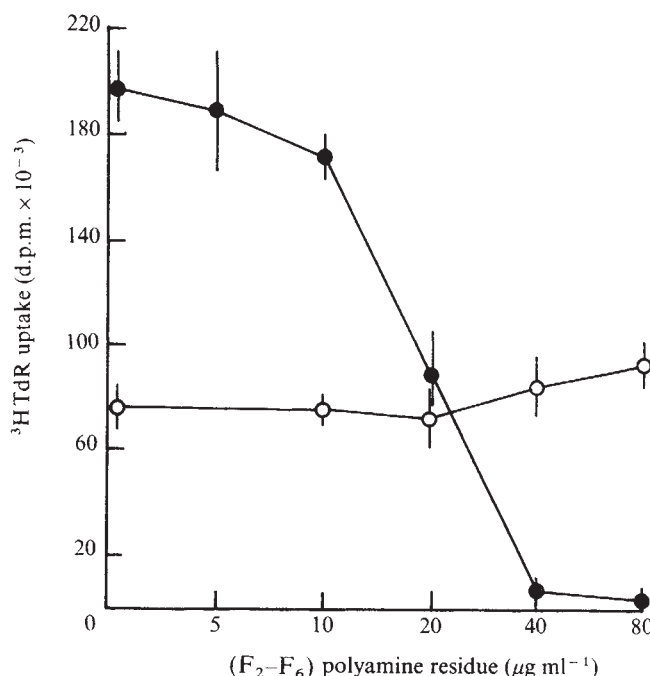
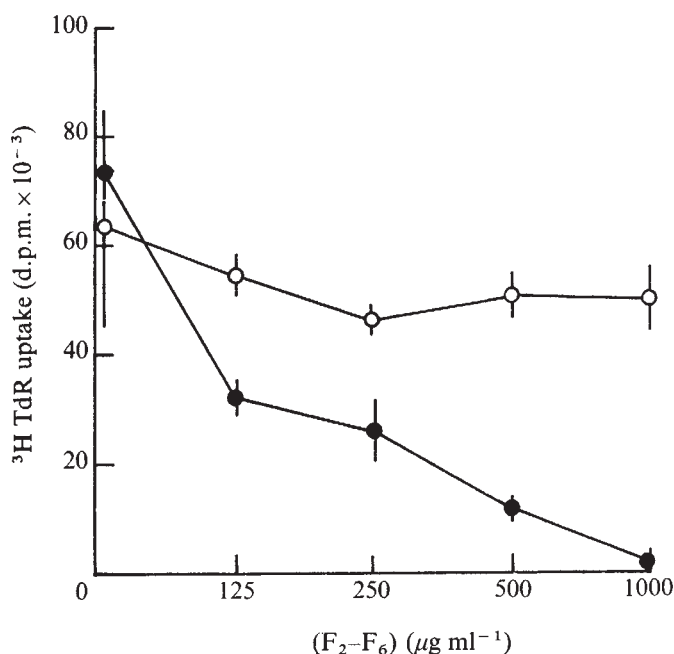


Fig. 2 Inhibitory activity of polyamine residue separated from thymic fraction (F₂-F₆) on uptake of ³HTdR by PHA-transformed lymphocytes in foetal calf serum (●) and in human serum (○). Culture details are presented in Table 1. Each point represents the mean d.p.m. of triplicate cultures $\pm$ s.d. (background subtracted).

polyamines. Fraction (F₂-F₆) was subjected to the polyamine extraction procedure of Inoue and Mizutani¹⁰. The hydrolysed residue obtained potentially inhibited lymphocyte transformation in foetal calf serum ($ID_{50}=20 \mu\text{g ml}^{-1}$) but not in human serum (Fig. 2). Analysis by gas-liquid chromatography (GLC) of the trifluoroacetylated residue using the method of McGregor *et al.*¹¹ confirmed that the residue contained roughly 20% spermine together with a trace of spermidine. On the basis of its spermine content, the residue had an ID_{50} of about $4.0 \mu\text{g ml}^{-1}$, reasonably close to that of authentic spermine. The loss of apparent tissue specificity on extraction of spermine from fraction (F₂-F₆) suggests that the spermine may exist in (F₂-F₆) as a tightly bound complex, with the carrier conferring the specificity. In addition, since both spermine and spermidine are present in normal tissues¹², the carrier itself showed a preference for spermine.

Finally, a series of experiments using ¹⁴C-spermine tetrahydrochloride were conducted, to study the extent of dialysis of spermine from the thymic fractions and from other materials. The essential finding was that 0.6% to 3.5% of spermine initially present could survive the extraction procedure (full details will be published elsewhere). Available data on polyamine concentrations in tissues¹³ show that this is quite sufficient to account for the amount isolated and identified in this work.

These results emphasise the difficulties associated with the isolation of an authentic lymphocyte chalone, but by no means rule out its existence. Polyamines have been implicated in growth processes¹⁴⁻¹⁶ and increased cellular levels are associated with optimal PHA-induced transformation of lymphocytes¹⁷. The activation of spermine, possibly with an amine oxidase, may thus represent a sensitive physiological control mechanism. The possibility that this mechanism is mediated by a larger, specific carrier molecule is not discounted.

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- ¹ Schafer, E. A. in *The Endocrine Organs* (Longmans Green, London, 1916).
- ² Bullough, W. S., Laurence, E. B., Iverson, O. H. & Eljgo, K. *Nature*, **214**, 578–580 (1967).
- ³ Houck, J. C. (ed.) in *Chalones* (North-Holland/American Elsevier, New York, 1976).
- ⁴ Houck, J. C., Attallah, A. M. & Lilly, J. R. *Nature*, **245**, 148–150 (1973).
- ⁵ Kiger, N., Florentin, I. & Mathé, G. in *Chalones: Concepts and Current Research*. National Cancer Institute Monograph 38, 135–141 (U.S. Dept Health, Education and Welfare, 1973).
- ⁶ Thornley, A. L. & Laurence E. B. *Int. J. Biochem.* **6**, 313–320 (1975).
- ⁷ Heidemann, E., Jung, A. & Wilms, K. *Klin. Wschr.* **54**, 221–226 (1976).
- ⁸ Lenfant, M., Privat de Garilhe, M., Garcia-Giralt, E. and Tempête, C. *Biochim. biophys. Acta* **541**, 106–117 (1976).
- ⁹ Byrd, W. J., Jacobs, D. M. and Amoss, M. S. *Nature* **267**, 621–623 (1977).
- ¹⁰ Inoue, H. & Mizutani, A. *Anal. Biochem.* **56**, 408–419 (1973).
- ¹¹ McGregor, R. F., Sharon, M. S., Atkinson, M. & Johnson D. E. *Prep. Biochem.* **6**, 403–419 (1976).
- ¹² Cohen, S. S. in *Introduction to the Polyamines*. (Prentice Hall, New Jersey, 1971).
- ¹³ Rosenthal, S. M. & Tabor, C. W. *J. Pharmac. exp. Therap.* **116**, 131–138 (1956).
- ¹⁴ Bachrach, U. in *Function of Naturally Occurring Polyamines*, 1–211 (Academic, New York, 1973).
- ¹⁵ Heby, O., Gray, J. W., Lindl, P. A., Marton, L. J. & Wilson, C. B. *Biochem. biophys. Res. Commun.* **71**, 99–105 (1976).
- ¹⁶ Fillingame, R. H., Jorstad, C. M. & Morris, D. R. *Proc. natn. Acad. Sci. U.S.A.*, **72**, 4042–4045 (1975).
- ¹⁷ Paukovits, W. R. *Cell Tissue Kinetics* **4**, 539–547 (1971).

Calcium ion produces graded changes in permeability of membrane channels in cell junction

THE permeability of the membrane channels in *Chironomus* salivary gland cell junction depends on the cytoplasmic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) (refs 1, 2). At the normal $[\text{Ca}^{2+}]_i$, $< 10^{-7}$ M, the channels are highly permeable to a wide range of molecular sizes; the upper size limit for peptide molecules is about 1,200–1,900 molecular weight (MW) (refs 3,4). When the $[\text{Ca}^{2+}]_i$ is elevated above 5×10^{-5} M in the junctional locale, the permeability falls drastically for all molecular species including the small inorganic ions^{2,5,6}. With elevations ranging up to about 5×10^{-5} M the channel permeability is reduced for the 330-MW fluorescein molecule with little or no detectable reduction of electrical coupling⁷. This suggested the interesting possibility that in this range the permeability change may be selective, that is, the permeability may fall for the larger molecular species but not for the small inorganic molecules carrying the electrical current. An alternative—that a fraction of the junctional channels closed unselectively (that is, completely)—could not be excluded, however, because the sensitivities of the methods for discriminating changes in the junctional permeabilities of the large and small molecular species were not equivalent; the measurements of electrical coupling, while quite sensitive to changes in junctional permeability in the low range of electrical coupling, are relatively insensitive at high levels of coupling⁷. Here we investigate this point by testing junctional permeability with fluorescent molecules of various sizes. In each test we use a pair of molecular species and compare their transit times during simultaneous diffusion in the same direction across the junction. If and only if the non-selective mechanism governs the Ca^{2+} -mediated permeability reduction, the transit for both species should be equally retarded, irrespective of size or structural differences between the probe molecules. We show that, at low $[\text{Ca}^{2+}]_i$,

elevation, the junctional permeability reduction is indeed selective and that the molecular size limit for junctional channel permeation changes in a graded manner.

A series of seven water-soluble fluorescent-labelled molecules ranging from 380 to 1,158 MW were used as permeability probes of the junction of *Chironomus* salivary gland cells (mid-fourth instar). These tracer molecules permeate the junctional membrane but not significantly the non-junctional membrane³ and, at the $[\text{Ca}^{2+}]$ used, their Ca binding is negligible and their (paper) electrophoretic mobility is unchanged. The tracer molecules were injected into the cells using micropipettes and a pneumatic pressure system². Permeabilities were tested by pair comparisons; each pair of tracers to be studied (Table 1) was injected as a mixture. In every pair, one tracer had a yellow-green fluorescent label of fluorescein isothiocyanate (FITC) or dansyl (DANS), and the other, a red fluorescent label of lissamine rhodamine B (LRB). Thus, the members in the pairs were easily distinguishable in the cells by means of appropriate barrier filters. The concentration ratio for a given pair was approximately constant and the fluorescence intensities were roughly matched. Only changes in transit time are of interest, however, so differences in absolute concentration and in detectability were of no consequence (so long as source concentration $\gg$ threshold for detection). The intracellular and transjunctional spread of the fluorescent tracers was observed and photographed directly in a darkfield microscope and/or scanned and videotaped through an image intensifier–television system². The tracers moved through the cytoplasm and the junction with velocities inversely related to their molecular weights; their arrival at the junction was marked by an abrupt fall in velocity³. Junctional permeabilities were measured as transit time, that is, the interval between the moment the tracer arrives at the junction and the moment it appears in the adjacent cell. Junctional transit speed in elevated $[\text{Ca}^{2+}]_i$ will be given relative to speed in Ca-free controls (determined at the same concentration ratio for a given pair); the relative transit speed (v), then, is defined as the ratio of the mean transit time of controls to transit time in the presence of Ca^{2+} . The experiments were carried out at room temperature, 22–25 °C.

Ca^{2+} buffered with citrate or mixed with ethyleneglycol-bis (β -aminoethyl ether)-*N*-*N'*-tetraacetic acid (EGTA) was injected together with the tracers or, in some cases, separately before the tracer injection. The free Ca^{2+} concentration in the injection solutions ranged from 10^{-4} to 10^{-2} M: this concentration had to be so high because Ca^{2+} is rapidly sequestered inside the cells and its concentration falls off precipitously with distance from the injection point. By the time the injected Ca^{2+} reaches the junction, some 50 μm away from our standard injection point (about cell centre), its concentration has fallen several orders of magnitude⁸. With the quantities and rates of the present Ca^{2+} injections, we expect, on the basis of earlier work in which the spatial distribution of $[\text{Ca}^{2+}]_i$ was determined using aequorin², that the $[\text{Ca}^{2+}]_i$ elevations in the junctional locale were in the range of 10^{-7} to 10^{-5} M.

The basic result was that, on elevation of $[\text{Ca}^{2+}]_i$ in this range, the transjunctional flow of the larger molecules was

Table 1 Tracer pairs

Pair no.		Molecular weight		Molecular weight
I	LRB (Leu) ₃ (Glu) ₂ OH	1,158	/DANS (Leu) ₃ (Glu) ₂ OH	849
II	LRB (Leu) ₃ (Glu) ₂ OH	1,158	/FITC (Glu) ₃ OH	794
III	LRB (Leu) ₃ (Glu) ₂ OH	1,158	/DANS (Glu) ₃ OH	640
IV	LRB (Leu) ₃ (Glu) ₂ OH	1,158	/DANS (Glu) ₂ OH	380
V	LRB (Gly) ₆ OH	901	/DANS (Glu) ₂ OH	380
VI	DANS (Leu) ₃ (Glu) ₂ OH	849	/LRB SO ₃ H	559
VII	FITC (Glu) ₃ OH	794	/LRB SO ₃ H	559

LRB, Lissamine rhodamine B; DANS, dansyl (dimethylaminonaphthalene sulphonyl); FITC, fluorescein isothiocyanate; Glu OH, glutamate; Leu OH, leucine; Gly OH, Glycine.

slowed more than that of the smaller ones and in some cases was even sensibly blocked while the small molecules were passing.

With Ca-free control solutions, containing EGTA or citrate, all seven tracer molecules passed through the junction with transit times ranging from a few seconds to about 1 min, depending on size. The transit times were the same as when EGTA or citrate was omitted³. With Ca-containing solutions the junctional transits were retarded, and in almost every test with pairs *III* to *VII* (Table 1) the retardation of the larger molecule (L) exceeded that of the smaller companion (S). Figure 1 presents the complete data for relative junctional transit speeds (v) of tracer pairs *III*, *IV*, *VI* and *VII*. The plots include also those cases with selective block of one molecule in a pair, invariably the larger ($v_L \approx 0$). Drawn in each graph is the line ($v_L/v_S = 1$) that would represent equal retardation of both tracers, namely non-selective retardation of junctional permeability. Nearly all points fall below the line. Application of the two-sample t test to the aggregate data of each graph, to compare the retarded junctional tracer movement with the control tracer movement, shows clearly that for all four tracer pairs the permeability reduction is selective; the confidence levels for $v_L/v_S < 1$ are 0.5, 5, < 0.5, and < 0.1% respectively for the pairs *III*, *IV*, *VI* and *VII*. A smaller number of experiments with *V* gave the same results; although only 6 trials were made, the confidence level was 3%. No selective junctional permeability reduction was found with the two pairs of the four largest molecules, pairs *I* and *II*; within the resolution of the method, the junctional transits of these molecules were equally retarded by $[Ca^{2+}]_i$ elevation. Eventually, at high $[Ca^{2+}]_i$, all seven molecules were blocked.

A significant feature of the results is the occurrence of selective block of junctional transit of the larger molecule in the pairs giving selective permeability reduction (two examples are

Fig. 1 Selective retardation of junctional transit by Ca^{2+} . Plotted are the relative junctional transit speeds (v) modified by Ca^{2+} for the small (S, abscissae) and large members (L, ordinates) of tracer pairs *III*, *IV*, *VI* and *VII*. Each value of v is the ratio of the mean junctional transit time in Ca-free controls to a single measurement of transit time in elevated $[Ca^{2+}]_i$. The subscripts give the molecular weights of the tracers. Each graph presents all data for a given tracer pair, including those of selective block (circled; $v_L \approx 0$). Squares denote repeated data points, with the multiplicity indicated. The line represents $v_S = v_L$, that is, equal (non-selective) retardation of junctional transit. The resultant confidence levels for $v_L < v_S$ are (*III*) 0.5, (*IV*) 5, (*VI*) < 0.5 and (*VII*) < 0.1%. (The numbers of controls and two other underlying statistics, the ratios of mean transit times (L/S) of the controls; s.e. were: (*III*) 5; 1.29 ± 0.27 ; (*IV*) 8; 2.67 ± 0.58 ; (*VI*) 5; 2.36 ± 0.54 ; (*VII*) 11; 1.18 ± 0.16 , respectively.)

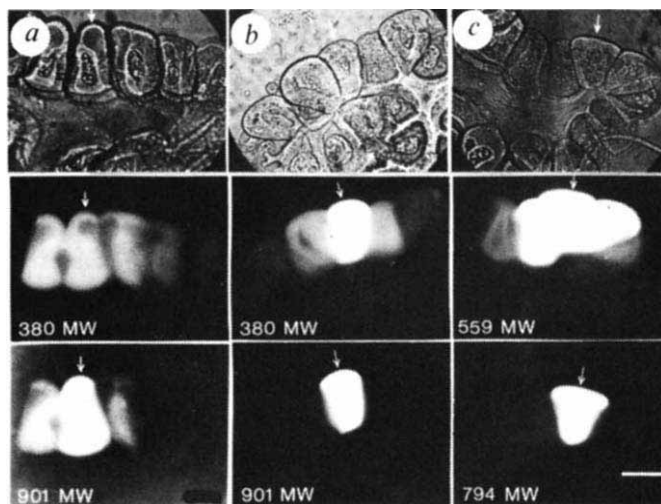
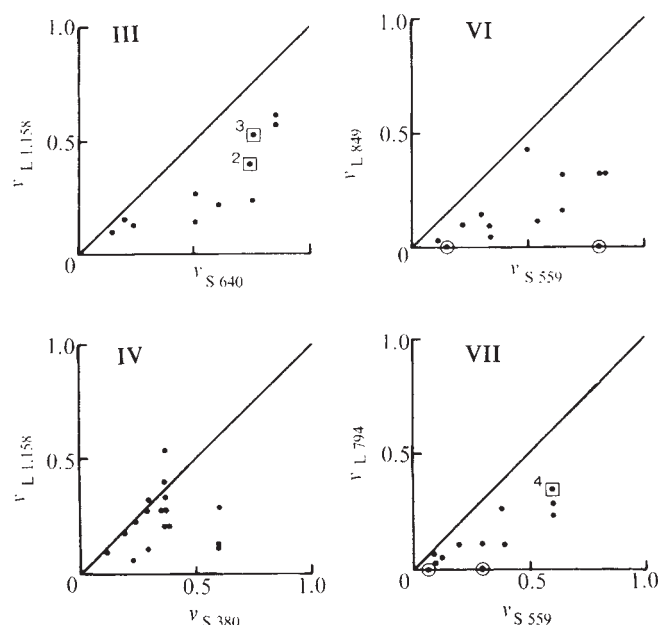


Fig. 2 Selective block of junctional transit. Permeability of junctional membrane channels is probed with pairs of fluorescently-labelled tracers. The fluorescence of one tracer in each pair is yellow-green and the other is red. The pairs are microinjected into a cell (arrow) together with Ca-buffers and the tracer fluorescence is photographed. Each series of photographs gives, from top to bottom, a view of the cells; the intracellular fluorescence of the smaller tracer molecule; that of the larger molecule (set apart by excitation and barrier filters.) The molecular weights on the photographs identify the tracer pairs. *a*, control; both molecules of the pair have passed from cell to cell. *b* and *c*, the molecules are injected together with Ca^{2+} ; passage of the larger molecule is blocked. The Ca-citrate ratios were: *a*, 0; *b*, 0.8; *c*, 1.0; citrate_{total}, 0.03 M. The photographs of the blocked tracers (*b* and *c*) were taken 5 min after those of their respective smaller companions; the interval injection-to-photography was in *b* and *c* 35-fold the control transit time. In *a* the photograph of the larger tracer was taken before that of the smaller tracer; the interval injection-to-photography for the larger tracer was 15-fold the control transit time. Calibration, 100 μ m.

illustrated in Fig. 2). This shows that the molecular weight limit for junctional permeation decreases on $[Ca^{2+}]_i$ elevation, and decreases in a graded fashion. If a stepwise decrease in molecular limit for junctional permeation is assumed, then, with the resolution afforded by the present probe repertoire, one may admit at least one gradation: one between 1,158 and 640 MW (or between 794 and 559). If we extrapolate now to the earlier result showing a dissociation between the junctional passage of the molecule of 330-MW (fluorescein) and electrical coupling, two further steps seem likely: one between 330 MW and the size of the smallest inorganic ion; and a third where even the latter ion is barred from junctional passage as the $[Ca^{2+}]_i$ concentration at the junction rises above about 5×10^{-5} M (refs 2,6). The actual grading may, of course, be finer and charge properties of the molecules may be additional contributing factors.

In seeking a controlling variable for the grading in junctional permeability, one is drawn to $[Ca^{2+}]_i$. Not only is this variable continuous, but at extreme values (10^{-7} M and $\sim 5 \times 10^{-5}$ M) it is associated with the extremes of junctional permeability, and an intermediate range is associated with intermediate permeabilities.

Seen in terms of the idea that the permeable junction is made of unit channels^{4,9-11}, the gradual decrease of the molecular size limit may be attributed to a gradual diminution of effective channel size on $[Ca^{2+}]_i$ elevation. The junction would behave like a sieve in which the unit mesh can be controlled by Ca^{2+} . Alternatively, the junction might contain channels of different size and Ca sensitivity. Here the result would be accounted for by an all-or-none closure of channels with Ca^{2+} sensitivities directly related to channel size.

As to the mechanism whereby Ca^{2+} affects the channels, it is plausible that this ion binds to junctional membrane causing a change in the channel's fixed charge or molecular conformation that reduces its effective size (by a decrease in the size of the membrane channels or by their misalignment on the two

membranes)^{4,7}. There is electron microscopic and spectroscopic evidence for Ca deposits at junctional membranes¹²⁻¹⁴ and for changes in size and packing of membrane particles in 'gap' junction in conditions where $[Ca^{2+}]_i$ is expected to have risen¹⁵.

The graded control of junctional permeability by Ca^{2+} offers in principle a powerful mechanism for physiological regulation of intercellular communication; it provides a means for selective transmission of intercellular molecular signals. A case in point may be in embryonic development where, as proposed earlier¹⁶, selective uncoupling of molecular signals could provide an economical means for producing stable cellular differentiations. In fact, for early embryos, there is evidence that fluorescein may not pass between electrically coupled cells¹⁷⁻¹⁹. If this phenomenon turns out to be due to a truly selective restriction of permeability (rather than due to a non-selective reduction in the number of channels; see above), it will be interesting to see whether the $[Ca^{2+}]_i$ is elevated at this developmental stage.

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- ¹ Rose, B. & Loewenstein, W. R. *Nature* **254**, 250-252 (1975).
- ² Rose, B. & Loewenstein, W. R. *J. Membrane Biol.* **28**, 87-119 (1976).
- ³ Simpson, I., Rose, B. & Loewenstein, W. R. *Science* **195**, 294-296 (1977).
- ⁴ Loewenstein, W. R. *Cold Spring Harb. Symp. quant. Biol.* **40**, 49-63 (1975).
- ⁵ Loewenstein, W. R., Nakas, M. & Socolar, S. J. *J. gen. Physiol.* **50**, 1865-1891 (1967).
- ⁶ Oliveira-Castro, G. M. & Loewenstein, W. R. *J. Membrane Biol.* **5**, 51-77 (1971).
- ⁷ Déléze, J. & Loewenstein, W. R. *J. Membrane Biol.* **28**, 71-86 (1976).
- ⁸ Rose, B. & Loewenstein, W. R. *Science* **190**, 1204-1206 (1975).
- ⁹ Loewenstein, W. R. *Ann. N. Y. Acad. Sci.* **137**, 441-472 (1966).
- ¹⁰ Goodenough, D. *Cold Spring Harb. Symp. quant. Biol.* **40**, 37-43 (1975).
- ¹¹ Gilula, N. B. & Epstein, M. L. *Symp. Soc. exp. Biol.* **30**, 257-272 (1976).
- ¹² Oschman, J. L. & Wall, B. J. *J. Cell Biol.* **55**, 58-71 (1972).
- ¹³ Oschman, J. L. & Wall, B. J. in *Transport Mechanisms in Epithelia* (eds Ussing, H. H. & Thorn, N. A.), 392-402 (Munsgaard, Copenhagen, 1974).
- ¹⁴ Larsen, W. J. *Cell Biol.* **67**, 801-813 (1975).
- ¹⁵ Peracchia, C. & Dulhunty, A. F. *J. Cell Biol.* **70**, 419-439 (1976).
- ¹⁶ Loewenstein, W. R. *Dev. Biol.* **19** Suppl. 2, 151-183 (1968).
- ¹⁷ Slack, C. & Palmer, J. F. *Exp. Cell Res.* **55**, 416-419 (1969).
- ¹⁸ Tupper, J. & Saunders, J. W. *Dev. Biol.* **27**, 546-554 (1972).
- ¹⁹ Bennett, M. V. L., Pappas, G. D. & Spira, M. E. *Dev. Biol.* **29**, 419-435 (1972).

Selective inhibition of thromboxane A_2 biosynthesis in blood platelets

SAMUELSSON's group^{1,2} showed that human platelets convert arachidonic acid and prostaglandin (PG) endoperoxides³ to thromboxane A_2 (TXA₂). TXA₂ strongly aggregates platelets¹ and contracts vascular strips^{1,4,5}. Vane's group^{5,6} has identified TXA₂ synthetase in platelet microsomes—this enzyme generates TXA₂ from PG endoperoxides. Aspirin-like drugs inhibit generation of PGs (refs 7, 8) and TXA₂ (ref. 9) in platelets at the early stage of cyclooxygenation of arachidonic acid^{10,11}. It is through this activity that aspirin, indomethacin, naproxen and other acidic anti-inflammatory drugs are anti-aggregatory^{11,12}—they have little or no effect on generation of TXA₂ from PG endoperoxides⁶. We report here that the non-acidic anti-inflammatory compound 2-isopropyl-3-nicotinyl-indole (L 8027) (refs 13, 14) is a selective TXA₂ synthetase inhibitor in platelets and a potent anti-aggregatory agent.

The inhibitory effect of L 8027 on the PG synthetase system was evaluated in bovine (BSVM) and ram (RSVM) seminal vesicle microsomal preparations. Fresh BSVM (refs 15, 16) or lyophilised RSVM (ref. 17) (2-3 mg ml⁻¹) were incubated with sodium arachidonate (33 μ M) in 66 mM phosphate buffer pH 8.0 for 5 min at 37 °C or at 30 °C, respectively. The incubation mixture with BSVM also contained glutathione (166 μ M) and hydroquinone (45 μ M). L 8027 (1-40 μ M) was pre-

incubated with the microsomal preparations for 6 min before the substrate was added. The activity of cyclo-oxygenase in RSVM was evaluated by polarographic measurement of the oxygen uptake¹⁸. The activity of PG synthetase system in BSVM and in RSVM was evaluated by bioassay of the PG-like product¹⁶. In BSVM 80-90% of the bioassayed PG-like activity was PGE₂ (ref. 13). The biologically active products which were formed from arachidonic acid by RSVM in the absence of cofactors were not separated by chromatography. The concentrations of L 8027 required to inhibit the enzymic activity by 50% (IC₅₀) were: 5.9 μ M in BSVM, 17 μ M in RSVM (as measured by bioassay) and 13 μ M in RSVM (as measured by oxygen consumption). Thus we found L 8027 to be 60-180 times less potent than indomethacin and 10-30 times more potent than aspirin as a PG synthetase inhibitor. This is in agreement with the data of Deby *et al.*¹³ on the relative anti-PG synthetase potency of L 8027.

TXA₂ synthetase activity in platelet microsomes was evaluated by an indirect method. The PG endoperoxide generating system of RSVM (ref. 3) was coupled⁶ to TXA₂ synthetase of horse platelet microsomes (HPM) (ref. 5). The biological activities of PG endoperoxides and TXA₂ were differentially assayed on a strip of rabbit mesenteric artery which is relaxed by PG endoperoxides and contracted by TXA₂ (ref. 19). Other bioassay organs used were rabbit aortic strip²⁰ and rabbit vena cava¹⁹ (both contracted by TXA₂ and by PG endoperoxides), rat stomach strip²¹ and rat colon²². Rat stomach strip is contracted by PEG₂, PGF_{2 α} , PG endoperoxides and TXA₂ in a decreasing order of potency⁵. Rat colon is not sensitive to TXA₂ and PG endoperoxides (up to 100 ng), but is contracted by PGF_{2 α} and PGE₂. The assay tissues were superfused in cascade²³ with Krebs bicarbonate (3 ml per min, 37 °C), containing a mixture of combined antagonists²⁴ and indomethacin (1 μ g ml⁻¹). In three experiments the concentrations of L 8027 needed for 50% inhibition of TXA₂ synthetase in HPM were 0.25 μ M, 0.3 μ M and 0.8 μ M. Thus L 8027 was 16-52 times more potent as an inhibitor of TXA₂ synthetase in HPM than as inhibitor of cyclooxygenase in RSVM.

In rabbit platelet homogenates L 8027 at concentrations of 0.3-1 μ M also inhibited TXA₂ generation from arachidonic acid (three experiments as in Fig. 1). At the same time L 8027 hardly influenced PG generation by platelet homogenates as shown by contractions of rat colon (Fig. 1).

The most striking inhibitory effect of L 8027 on TXA₂ generation, however, occurred in aggregating platelets. Aggregation of platelets in 1 ml of fresh citrated rabbit platelet-rich plasma (PRP) was monitored in a Chrono-Log aggregometer. Aggregation was induced by sodium arachidonate (AA, 25-200 μ M) or by adenosine diphosphate (ADP, 10 μ M). L 8027 was preincubated with PRP at 37 °C for 6 min before an aggregating agent was added. Sixty seconds later a 100- μ l aliquot of PRP was withdrawn and immediately bioassayed on a strip of rabbit mesenteric artery for TXA₂. In 10 PRP the threshold pro-aggregatory effect of AA (25-70 μ M) was completely abolished by L 8027 at concentrations of 40 pM-10 nM. Anti-aggregatory concentrations of L 8027 mounted up to 0.1-1.0 μ M in parallel to an increase in the pro-aggregatory concentrations of AA (100-200 μ M). These anti-aggregatory concentrations of L 8027 (40 pM to 1 μ M) also dramatically suppressed the release of TXA₂ from platelets by AA. In eight PRP the ADP-induced aggregation was not inhibited by L 8027 at concentrations up to 40 μ M. ADP-induced aggregation was not associated with the detectable release of TXA₂.

The selective inhibition of TXA₂ synthetase by L 8027 in horse platelet microsomes and in rabbit platelet homogenates explains the potent anti-aggregatory effect of L 8027 on rabbit platelets. But, high anti-aggregatory potency of L 8027 against the threshold pro-aggregatory concentrations of arachidonate as well as an instant drop of this potency in parallel to an increase in concentration of the pro-aggregatory agent indicates that anti-TXA₂ synthetase activity of L 8027 is strongly dependent on the substrate concentration. It could be that L

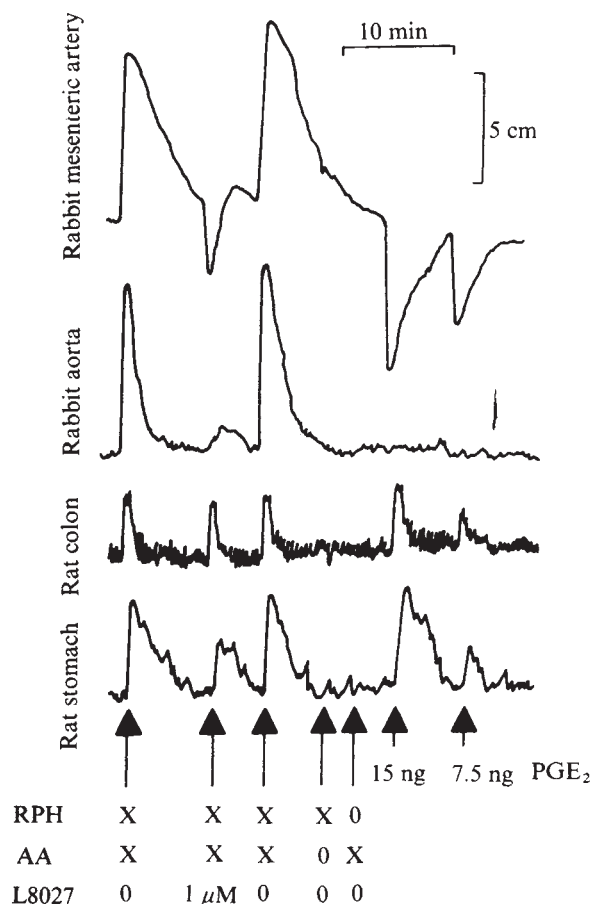


Fig. 1 Bioassay of thromboxane A_2 and prostaglandins generated during an incubation of arachidonic acid (AA) with rabbit platelet homogenate (RPH). Platelets were obtained from 40 ml of rabbit platelet rich plasma and resuspended in 5 ml of 50 mM Tris buffer pH 7.4. The suspension was subjected to thawing and freezing three times and centrifuged at 5000g for 15 min to remove cell debris⁹. 200 μ l of the supernatant (RPH) and 300 μ l of AA (300 μ M) were mixed and kept on ice. After 3 min an aliquot of 100 μ l was bioassayed. In one experiment L 8027 (1 μ M) was preincubated with RPH for 3 min at 22 °C before AA was added. The assay organs (rabbit mesenteric artery, rabbit aorta, rat colon and rat stomach) were calibrated with 15 ng and 7.5 ng of PGE₂. Presence or absence of each component of the incubation mixture is marked with X or 0, respectively. L 8027 inhibited the generation of TXA₂ by RPH (contractile response of vascular strips were either inverted or suppressed) and had little effect on prostaglandin generation (contractions of rat colon remained substantially not changed).

8027 is a competitive, reversible inhibitor of TXA₂ synthetase and therefore an IC₅₀ value is not an appropriate measure for its anti-enzymic activity. In our experiments with platelet microsomes we were not able to quantify the amount of PG endoperoxides in the incubation mixture and to calculate K_i value for L 8027. Inability of L 8027 to inhibit the ADP-induced platelet aggregation is in favour¹¹ of the proposed mechanism of anti-aggregatory action of L 8027.

In summary, in our experimental conditions L 8027 at 1 μ M significantly inhibits the generation of TXA₂ by platelet microsomes, by platelet homogenates and by platelet rich plasma. The same concentration of L 8027 does not influence the formation of PG-like material by BSVM, by RSVM and by platelet homogenates (Fig. 1) or the activity of cyclo-oxygenase in RSVM. In our preliminary experiments L 8027 (1 μ M) did not inhibit the spontaneous formation of prostacyclin (PGX, PGI₂) by the incubated rings of rabbit mesenteric arteries²⁷⁻²⁹. Therefore we conclude that L 8027 at concentrations of 1 μ M and lower is a selective inhibitor of TXA₂ generation in blood platelets.

Aspirin, indomethacin and other acidic cyclo-oxygenase

inhibitors are all being considered as anti-platelet drugs¹², though their pharmacological effects and side effects are of a broad spectrum. We suggest that non-acidic L 8027 which is a potent inhibitor of TXA₂ generation in platelets, can compete with cyclo-oxygenase inhibitors in the treatment of thromboembolic diseases.

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- Hamberg, M., Svensson, J. & Samuelsson, B. *Proc. natn. Acad. Sci. U.S.A.* **72**, 2994-2998 (1975).
- Hamberg, M., Svensson, J. & Samuelsson, B. *Advances in Prostaglandin and Thromboxane Research 1* (eds Samuelsson, B. & Paoletti, R.) 19-27 (Raven, New York, 1976).
- Hamberg, M. & Samuelsson, B. *Proc. natn. Acad. Sci. U.S.A.* **70**, 899-903 (1973).
- Svensson, J., Hamberg, M. & Samuelsson, B. *Acta physiol. scand.* **94**, 222-228 (1975).
- Needleman, P. et al. *Nature* **261**, 558-560 (1976).
- Moncada, S., Needleman, P., Bunting, S. & Vane, J. R. *Prostaglandins* **12**, 323-336 (1976).
- Vane, J. R. *Nature new Biol.* **231**, 233-235 (1971).
- Smith, J. B. & Willis, A. L. *Nature new Biol.* **231**, 235-237 (1971).
- Green, K., Hamberg, M. & Samuelsson, B. *Advances in Prostaglandin and Thromboxane Research 1* (eds B. Samuelsson, B. & Paoletti, R.) 47-58 (Raven, New York, 1976).
- Flower, R. J. *Pharmac. Rev.* **26**, 33-67 (1974).
- Smith, J. B. & Macfarlane, D. E. *The Prostaglandins 2* (ed. Ramwell, P. W.) 293-343 (Plenum, New York, 1974).
- Mustard, J. F. & Packham, M. A. *Drugs* **9**, 19-76 (1975).
- Deby, C., Descamps, M., Binon, F. & Bacq, Z. M. *C. r. Seanc. Soc. Biol.* **165**, 2465-2468 (1971).
- Colot, M. et al. *Thérapie* **28**, 775-798 (1973).
- Takeguchi, C., Kohno, E. & Sih, C. J. *Biochemistry* **10**, 2372-2376 (1971).
- Gryglewski, R. J. *Prostaglandin Synthetase Inhibitors* (eds Robinson, H. J. & Vane, J. R.) 33-52 (Raven, New York, 1974).
- Bergström, S., Danielsson, H. & Samuelsson, B. *Biochim. biophys. Acta* **90**, 170-180 (1964).
- Lands, W. E. M., Lee, P. & Smith, W. *Ann. N. Y. Acad. Sci.* **180**, 107-122 (1971).
- Bunting, S., Moncada, S. & Vane, J. R. *Proc. Br. pharmac. Soc.* April, 1976, p.48.
- Piper, P. J. & Vane, J. R. *Nature* **223**, 29-35 (1969).
- Vane, J. R. *Br. J. Pharmac.* **12**, 344-349 (1957).
- Regoli, D. & Vane, J. R. *Br. J. Pharmac.* **23**, 351-359 (1964).
- Vane, J. R. *Br. J. Pharmac.* **23**, 369-373 (1964).
- Gilmore, N., Vane, J. R. & Wyllie, J. H. *Nature* **218**, 1135-1140 (1968).
- Bundy, G. L. *Tetrahedron Lett.* **24**, 1957-1960 (1975).
- Malmsten, C. *Life Sci.* **18**, 169-176 (1976).
- Moncada, S., Gryglewski, R., Bunting, S. & Vane, J. R. *Nature* **263**, 663-665 (1976).
- Gryglewski, R., Bunting, S., Moncada, S., Flower, R. J. & Vane, J. R. *Prostaglandins* **12**, 685-713 (1976).
- Moncada, S., Gryglewski, R., Bunting, S. & Vane, J. R. *Prostaglandins* **12**, 715-733 (1976).

Effects of PGD₂ on canine renal function

IN many tissues, especially the mammalian kidney, prostaglandin D₂ (PGD₂) is a significant product in the metabolism of the bisenoic prostaglandin endoperoxides^{1,2}. PGD₂ was originally reported to have negligible biological activity¹; but, subsequent workers noted that it produces vascular effects in several species^{3,4}. Although the effects in the kidney of the precursor, arachidonic acid, and other prostaglandins (PGs)⁵⁻⁷ have attracted much interest, the effects of PGD₂ have received little attention. Since PGD₂ may be an important product of arachidonate metabolism in the kidney^{8,9}, the renal response to an infusion of PGD₂ in the dog was examined and compared with the response to an infusion of PGE₂. Whereas both PGs produced an increase in renal blood flow, only PGE₂ caused a diuresis and natriuresis. This suggests that PGD₂ in the kidney is primarily involved in the control of renal haemodynamics, and does not participate in the regulation of fluid and electrolyte excretion.

Table 1 shows the renal effects of the infusion of PGD₂ and PGE₂. The infusion of either prostaglandin led to a pronounced increase in total renal blood flow (RBF). A marked diuresis and natriuresis accompanied the increase

in RBF produced by the infusion of PGE₂, but not PGD₂. The glomerular filtration rate (GFR) was not affected by the infusion of PGE₂, but there was a significant reduction in this parameter during the infusion of PGD₂. Both PGs significantly enhanced the levels of plasma renin activity in the renal vein (Table 1). The enhancement of RBF induced by the infusion of PGD₂ was caused by an elevation of blood flow to both the outer and inner cortical regions (Fig. 1). No significant alterations were observed in the contralateral kidney, the mean systemic blood pressure or the heart rate during the infusion of either prostaglandin.

Intrarenal infusion of PGD₂ and PGE₂ produced a similar degree of increase in total RBF. Although intrarenal blood flow during the infusion of PGE₂ was not reported in this study, Chang *et al.*⁷ observed that an intrarenal infusion of PGE₂ in the dog promoted an elevation of blood flow to the outer and inner cortical regions. In two experiments in which intrarenal blood flow was measured, the intrarenal infusion of PGE₂ produced alterations in intrarenal blood flow identical to those cited by Chang and co-workers⁷. The intrarenal infusion of PGD₂ also promoted an enhancement of outer and inner cortical blood flows confirming results obtained by Friesinger *et al.*¹⁰. These changes in intrarenal blood flow are similar to those produced by many vasodilators, but differ from the alterations produced by the infusion of the prostaglandin precursor, sodium arachidonate (unpublished observation of P.M.B. *et al.*). When sodium arachidonate is infused intrarenally there is an elevation of inner cortical blood flow, but no concomitant increase in the blood flow to the outer cortex.

As mentioned, the enhancement of RBF induced by the infusion of PGE₂ was accompanied by a marked diuresis and natriuresis, but infusion of PGD₂ had no effect on fluid and electrolyte excretion rates although it did induce changes in renal haemodynamics similar to those produced

by PGE₂. Oelz *et al.*¹¹ have reported that intrarenal infusion of PGD₂ produced a natriuresis and diuresis in addition to an enhancement of RBF. (Since their data appeared in abstract form it is difficult at present to provide an explanation for the discrepancy between our work and theirs.) During PGD₂ infusion in the present study there was a reduction in GFR, which could have offset any direct tubular effects of PGD₂. This is unlikely, however, since in subsequent studies we have found that a lower dose of PGD₂ (0.04 µg per kg body weight per min) which produced effects qualitatively identical to those seen with the higher dose of PGD₂ (namely an increase in RBF without an accompanying diuresis or natriuresis) did not reduce the GFR. The absence of a natriuresis and diuresis during PGD₂ infusion could not have been due to a reduction in the renal perfusion pressure, since there was no change in the mean systemic blood pressure during the infusion of PGD₂.

It is possible that PGE₂ promoted a diuresis and natriuresis because of a direct tubular inhibition of fluid and electrolyte transport. Several groups have observed that the renal prostaglandin system inhibits the action of vasopressin on the collecting duct^{12,13}, and in addition, in the isolated perfused rabbit cortical collecting tubule PGE₂ has recently been shown to inhibit directly the reabsorption of sodium¹⁴. The finding that PGD₂ did not alter the electrolyte and fluid excretion rates is somewhat unusual, since most renal vasodilators produce a natriuresis and diuresis which accompanies an increase in RBF^{15,16}. One exception to this is secretin, which was found to behave in a similar fashion to PGD₂ when infused into the renal artery¹⁷. It exhibited no effect on electrolyte and fluid excretion rates while increasing RBF, and when administered in low dosage there was no significant change in the GFR.

The infusion of both PGD₂ and PGE₂ led to an elevation

Table 1 Effects of PGD₂ and PGE₂ on canine renal function

Infusion periods	RBF (ml min ⁻¹ g ⁻¹) (n = 5)	V (µl min ⁻¹ g ⁻¹) (n = 8)	U _{Na} V (µEq min ⁻¹ g ⁻¹) (n = 8)	FE _{Na} (n = 8)	C _{H₂O} (µl min ⁻¹ g ⁻¹) (n = 8)	GFR (ml min ⁻¹ g ⁻¹) (n = 8)	RVPR (ng ml ⁻¹ h ⁻¹) (n = 6)
Control	3.2 ± 0.4	15 ± 5	0.9 ± 0.3	0.9 ± 0.3	-3 ± 4	0.65 ± 0.04	8.7 ± 3.9
PGD ₂	7.5 ± 1.4*	15 ± 4	0.8 ± 0.2	1.0 ± 0.3	0 ± 4	0.54 ± 0.04*	20.0 ± 3.8*
Control	3.6 ± 0.4	16 ± 5	0.9 ± 0.3	1.0 ± 0.3	0 ± 3	0.58 ± 0.05	12.3 ± 4.1
	(n = 5)	(n = 7)	(n = 7)	(n = 7)	(n = 7)	(n = 7)	(n = 5)
Control	4.1 ± 0.7	16 ± 5	0.7 ± 0.3	0.7 ± 0.3	0 ± 4	0.60 ± 0.06	10.5 ± 4.0
PGE ₂	8.0 ± 1.5*	40 ± 12*	2.3 ± 0.7*	2.5 ± 0.6*	13 ± 8*	0.68 ± 0.15	21.2 ± 7.1*
Control	3.6 ± 0.7	11 ± 2	0.9 ± 0.3	1.1 ± 0.3	-5 ± 2	0.58 ± 0.08	13.5 ± 5.9

Values for the right kidney are given as the mean ± the standard error. n, Number of animals; RBF, renal blood flow; V, urinary flow rate; U_{Na}V, urinary sodium excretion rate; C_{H₂O}, free water clearance rate; GFR, glomerular filtration rate; FE_{Na}, fractional excretion of sodium; RVPR, renal vein plasma renin activity; * P < 0.05. Mean values for RBF and RVPR were not obtained in all nine animals because of inaccurate flow readings in two and the inability to advance a catheter into the right renal vein for blood collections in two others. Mongrel dogs of either sex were fasted for 18 h before surgery and allowed free access to water. Anaesthesia was induced by intravenous pentobarbital (30 mg per kg body weight). An indwelling polyethylene catheter was inserted into the right femoral artery for monitoring systemic blood pressure and collection of blood samples. A catheter was introduced into the left femoral artery proximal to the flowprobe and advanced into the left ventricle for injection of radionuclide labelled microspheres (15 ± 5 µm, New England Nuclear). A catheter was also placed in the left femoral vein and advanced into the right renal vein for collection of renal venous blood. A fourth catheter was introduced into the right femoral vein for the infusion of inulin used, to measure GFR. A suprapubic midline abdominal incision was made and both ureters were cannulated. A right retroperitoneal flank incision was made, and a hydraulic cuff and electromagnetic flowprobe were placed around the renal artery. Renal blood flow was monitored by a sine wave, non-occluding electromagnetic flowmeter (Biotronex Laboratories). The hydraulic cuff was used to establish and verify zero renal blood flow. A 25-gauge needle was inserted into the right renal artery proximal to the flowprobe and hydraulic cuff, and normal saline was infused at 0.5 ml min⁻¹. A period of 1 h was allowed for stabilisation before the commencement of the experiment. The study comprised five periods of 15 min duration each. Three 5-min urine collections were obtained during each period. Midway through each period, arterial samples for inulin, osmolality and sodium measurements and renal vein samples for renin determinations were collected. After the first 15-min control period, either PGD₂ (0.4–0.8 µg kg⁻¹ min⁻¹) or PGE₂ (0.4 µg kg⁻¹ min⁻¹), was infused into the renal artery for 15 min. Twenty minutes were allowed for renal function to return to control levels and then a second 15-min control period was begun. A 15-min infusion of either PGD₂ or PGE₂ (whichever had not been used before) immediately followed the second control period. The sequence of infusion of PGD₂ and PGE₂ was varied. A final 15-min control period was initiated 20 min after completion of the second PG period. Three bolus injections of differently labelled radionuclide microspheres (⁸⁵Sr, ¹⁴¹Ce, ⁵¹Cr) were administered immediately following the blood collections in the control period before infusion of PGD₂, during the infusion of PGD₂ and in the control period immediately following PGD₂ infusion. At the end of the fifth period, the animals were killed and the kidneys were removed, weighed and sectioned according to the method of Slotkoff *et al.*²⁰. The sections were counted for radioactivity, to determine intrarenal blood flow. Renin levels were measured by radioimmunoassay. The level of significance was determined by the paired t test.

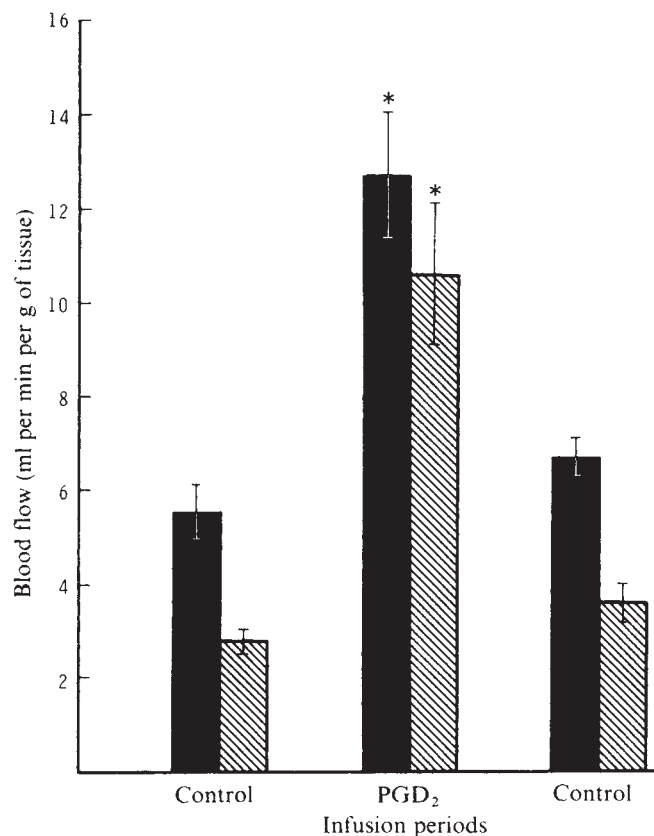


Fig. 1 The effect of PGD₂ on outer cortical and inner cortical blood flows (5 animals). Values for the right kidney are given as the mean $\pm$ the standard error. Outer cortical blood flow is represented by the solid bars and inner cortical blood flow is represented by the striped bars. * $P < 0.05$.

in renal venous plasma renin activity (PRA). It has been shown in an *in vitro* rabbit kidney preparation that arachidonate and several of the PGs directly stimulate renin release¹⁸. Arachidonic acid has also been shown to promote the *in vivo* release of renin in several species^{5,19}.

This study has shown that unlike PGE₂ and other vasodilators, an intrarenal infusion of PGD₂ alters renal haemodynamics without producing concomitant changes in electrolyte and fluid excretion. An infusion of PGD₂ also seems to stimulate renin release; whether this represents a direct action or is consequent to renal haemodynamics cannot yet be answered.

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- Nugteren, D. H. & Hazelhof, E. *Biochim. biophys. Acta* **326**, 448–461 (1973).
- Helmer, M. & Lands, W. E. M. *J. biol. Chem.* **251**, 5575–5579 (1976).
- Hamberg, M., Hedqvist, P., Strandberg, K., Svensson, J. & Samuelsson, B. *Life Sci.* **16**, 451–462 (1975).
- Armstrong, J. M., Boura, A. L. A., Hamberg, M. & Samuelsson, B. *Eur. J. Pharmacol.* **39**, 251–258 (1976).
- Bolger, P. M., Eisner, G. M., Ramwell, P. W. & Slotkoff, L. M. *Nature* **259**, 244–245 (1976).
- Tannenbaum, J., Splawinski, J. A., Oates, J. A. & Nies, A. S. *Circulat. Res.* **36**, 197–203 (1975).
- Chang, L. C. T., Splawinski, J. A., Oates, J. A., & Nies, A. S. *Circulat. Res.* **36**, 204–207 (1975).
- Blackwell, G. J., Flower, R. J. & Vane, J. R. *Biochim. biophys. Acta* **398**, 178–190 (1975).

- Tai, H.-H., Tai, C. L. & Hollander, C. S. *Biochem. J.* **154**, 257–264 (1976).
- Friesinger, G. C. *et al. Clin. Res.* **25**, 41A (1977).
- Oelz, O., Sweetman, B. J., Oates, J. A., Nies, A. S. & Data, J. L. *Pharmacologist* **18**, 163 (1976).
- Grantham, J. & Orloff, J. *J. clin. Invest.* **47**, 1154–1161 (1968).
- Anderson, R. J., Berl, T., McDonald, K. M., & Schrier, R. W. *J. clin. Invest.* **56**, 420–426 (1975).
- Stokes, J. B. & Kokko, J. P. 9th Ann. Mtg Am. Soc. Nephrol. **114** (1976).
- Daugharty, T. M., Belleau, L. J., Martino, J. A. & Earley, L. E. *Am. J. Physiol.* **215**, 1442–1447 (1968).
- Baer, P. & Navar, L. *Kidney Int.* **4**, 12–21 (1973).
- Marchand, G. R., Hubel, K. A. & Williamson, H. E. *Proc. Soc. exp. Biol. Med.* **147**, 652–655 (1974).
- Weber, P. C. *et al. Circulat. Res.* **39**, 868–874 (1976).
- Larsson, C., Weber, P., & Anggard, E. *Eur. J. Pharmacol.* **28**, 391–394 (1974).
- Slotkoff, L. M., Logan, A., Jose, P., D'Avella, J. & Eisner, G. M. *Circulat. Res.* **28**, 158–166 (1971).

Elevated serum levels of 1 α , 25-dihydroxycholecalciferol in lactating rats

LACTATION represents a challenge to regulation of calcium metabolism. Because of the high rate of calcium transfer to the milk, the lactating rat consuming a commercial stock diet maintains a reduced serum Ca level¹. The increased need for Ca seems to be met primarily by increased intestinal absorption of Ca (ref. 1). Since vitamin D is important for regulation of Ca metabolism, particularly during lactation², and since the circulating level of the active metabolite 1 α ,25-dihydroxyvitamin D₃ (1 α ,25-(OH)₂D₃) seems to be regulated according to Ca needs³, we determined by radioreceptor binding assay the serum levels of 1 α ,25-(OH)₂D₃ in vitamin D-fed lactating rats. We report here that these levels are two- to fourfold higher than those of non-lactating controls. Rats deprived of vitamin D during lactation fail to show elevated metabolite blood levels and develop marked hypocalcaemia, thus indicating the importance of the elevated blood level of 1 α ,25-(OH)₂D₃ for Ca metabolism during lactation.

1 α ,25-(OH)₂D₃ has been called the hormonal form of the vitamin⁴, because its formation in the kidney⁵ as well as its circulating level is apparently stringently controlled. Evidence for such regulation is the absence of an increase in the blood level of this metabolite in the face of a many-fold increase in the blood level of the precursor, 25-OHD₃, during chronic administration of excess vitamin D (refs 5, 6). Regulation of the blood level of 1 α ,25-(OH)₂D₃ may involve negative feedback of this metabolite on its synthesis in the kidney⁷. Factors that can stimulate renal 1 α -hydroxylation of 25-hydroxyvitamin D₃ (25-OHD₃) and cause an elevation of the 1 α ,25-(OH)₂D₃ level in the blood are hypophosphataemia^{3,8} and increased rate of secretion of parathyroid hormone^{3,8–10}, as well as hypocalcaemia independent of parathyroid hormone³.

Rats with litters of ten 6-d-old pups, and non-lactating females of the same age, were obtained from the Holtzman Company, where they had been maintained on a low vitamin D diet with 27% protein, 1.25–1.7% Ca and a Ca : P ratio of 1.5 : 1. On the seventh day of lactation, the adults were assigned randomly by weight to one of two dietary groups. One group received the basic diet consisting of 78% whole wheat flour, 14% casein, 4% corn oil, 2% Ca- and P-free salt mixture, 1.3% of vitamin D-free vitamin mixture (Nutritional Biochemicals) and 0.8% CaCO₃, with a final Ca and P content of 0.4% (–D diet). The other group received the basic diet to which had been added cholecalciferol (Sigma) at a concentration equal to that of Purina Laboratory Chow (5 IU per g of diet) (+D diet). Initially and at weekly intervals during the experiment, non-fasted adults were bled from the tail or by heart puncture and the adults and their litters were weighed. If the size of a litter fell below seven, the mother and pups were excluded from the experiment.

During a 4-d period (days 9–13) the food intake was measured and faeces were collected to determine the daily intake and rate of net absorption of Ca. Ca in food, faeces and serum was determined in a lanthanum chloride solu-

tion by atomic absorption spectrophotometry with a Perkin-Elmer 305 instrument. Phosphate (as inorganic phosphorus) was determined colorimetrically¹¹. Serum (3–4 ml per rat) was frozen for determination of $1\alpha,25-(\text{OH})_2\text{D}_3$ by a competitive radioreceptor binding assay which utilises the chick intestinal cytosol receptor and its association with chromatin. The $1\alpha,25-(\text{OH})_2\text{D}_3$ was first isolated from the serum as described by Hughes *et al.*³, and assayed according to recent modifications of the binding assay procedure¹². Triplicate assays were carried out on all samples, and the interassay variation was 10–15%. Studies with this assay show that vitamin D-deficient animals have undetectable levels of the hormone⁶ and measured circulating levels correlate with expected physiological modulations during dietary alterations of vitamin D (ref. 6), calcium and phosphate³.

Standard errors were calculated from analyses of variance of the data in each experiment and a one-tailed Student's *t* test was used to test significance of differences between mean values.

Table 1 contains the serum Ca levels on the seventh day, which marked the beginning of the dietary treatment, and the serum Ca, P and $1\alpha,25-(\text{OH})_2\text{D}_3$ levels on the 20th day. The seventh day values show that the lactating rats had significantly lower serum Ca levels than the non-lactating rats (experiments 2 and 3). Table 1 also shows that vitamin D-fed rats that had lactated for 20 d had elevated blood levels of $1\alpha,25-(\text{OH})_2\text{D}_3$. In experiment 2 the $1\alpha,25-(\text{OH})_2\text{D}_3$ blood levels of the lactating rats were over threefold higher than that of the non-lactating rats. The same lactating rats had significantly lower serum Ca and higher serum P levels than the non-lactating rats on the 20th day. When vitamin D was withheld from the diet for 2 weeks, lactating rats in experiments 1 and 2 had significantly lower serum $1\alpha,25-(\text{OH})_2\text{D}_3$ and Ca levels than vitamin D-fed lactating rats. This is in contrast to the vitamin D-deprived non-lactating rats (3) which showed no significant reduction in the serum levels of either $1\alpha,25-(\text{OH})_2\text{D}_3$ or Ca compared to their vitamin D-fed controls (experiment 3). Before the effect of vitamin D-deprivation (that is hypocalcaemia) was apparent in the rats in experiment 3 (days 9–13), intestinal absorption of Ca was 123 ± 2 mg per d or $62 \pm 4\%$ of Ca intake (mean $\pm$ s.e., $n=5$) for the lactating rats and 11 ± 2 mg per day or $15 \pm 3\%$ of Ca intake for the non-lactating rats. In another experiment (not shown) with rats that had been maintained on the +D diet and lactated for at least 3 weeks Ca absorption was comparable with that of the lactating rats in experiment 3, confirming the obser-

vation that calcium absorption is markedly elevated during lactation.

The present data represent the first demonstration of elevated serum levels of $1\alpha,25-(\text{OH})_2\text{D}_3$ during lactation. High levels of this hormone have also been found in young rats^{3,13} (as compared with adults) and in the laying hen in which the Ca needs also are increased¹². The serum $1\alpha,25-(\text{OH})_2\text{D}_3$ levels in the non-lactating rats in the present study are comparable with the levels reported for adult rats¹⁴.

The elevation of the $1\alpha,25-(\text{OH})_2\text{D}_3$ blood level in the vitamin D-fed lactating rats could have been due to the relative hypocalcaemia, which was clearly demonstrated, or to increased secretion of parathyroid hormone, which is a usual consequence of hypocalcaemia. Hypophosphataemia, on the other hand, can be ruled out as a stimulus in these rats since the serum phosphate values for the vitamin D-fed lactating rats were actually higher than those for the non-lactating controls. We should also consider the possibility that factors of importance for maintenance of milk secretion, such as thyroid and adrenal hormones and prolactin, also may contribute to elevation of the $1\alpha,25-(\text{OH})_2\text{D}_3$ blood level. In fact, prolactin injection in chicks has been shown to enhance both the activity of the renal 1α -hydroxylase¹⁵ and the circulating concentration of $1\alpha,25-(\text{OH})_2\text{D}_3$ (ref. 12).

While the present data are consistent with the idea that the elevated blood levels of $1\alpha,25-(\text{OH})_2\text{D}_3$ are essential for the maintenance of an increased rate of intestinal Ca absorption, it remains to be seen if a high rate of absorption also can be maintained in the vitamin D-deficient lactating rats perhaps by an action of parathyroid hormone^{16,17} or prolactin¹⁸ or other factors that may influence Ca absorption. Maintenance of serum Ca is apparently dependent on the elevated serum $1\alpha,25-(\text{OH})_2\text{D}_3$ level during lactation since severe hypocalcaemia ensued in vitamin D-deprived lactating rats that lacked the elevated $1\alpha,25-(\text{OH})_2\text{D}_3$ level. The level of $1\alpha,25-(\text{OH})_2\text{D}_3$ fell rapidly in the latter rats, if it can be assumed that this level was elevated during the first week of lactation before the vitamin D-deprivation. Since such deprivation did not affect this level in non-lactating rats, the present data are consistent with the idea that vitamin D metabolism is altered during lactation. In conclusion, it is suggested that elevation of the blood level of $1\alpha,25-(\text{OH})_2\text{D}_3$ takes place as a result of factors associated with the increased Ca demands of milk secretion, and that the elevated level of $1\alpha,25-(\text{OH})_2\text{D}_3$ is required for efficient regulation of Ca metabolism.

We thank Mrs Catherine Lane for technical assistance

Table 1 Serum calcium, phosphate and $1\alpha,25-(\text{OH})_2\text{D}_3$ levels in lactating and non-lactating rats

Experiment	Diet group	7th day of lactation		20th day of lactation		
		Ca (mg dl ⁻¹)	Ca (mg dl ⁻¹)	P (mg dl ⁻¹)	$1\alpha,25-(\text{OH})_2\text{D}_3$ (ng dl ⁻¹)	
1	Lactating	−D	9.8 $\pm$ 0.2	7.8 $\pm$ 0.4*	4.4 $\pm$ 0.3	5.8 $\pm$ 1.7
	Lactating	+D	9.6 $\pm$ 0.3	9.4 $\pm$ 0.5	5.9 $\pm$ 0.3	12.8 $\pm$ 1.2§
2	Lactating	−D	9.4 $\pm$ 0.1	7.6 $\pm$ 0.2*	3.7 $\pm$ 0.2	7.6 $\pm$ 0.6
	Lactating	+D	9.1 $\pm$ 0.1†	9.4 $\pm$ 0.2†	5.4 $\pm$ 0.2†	26.7 $\pm$ 0.6‡
	Non-lactating	+D	9.8 $\pm$ 0.1	10.1 $\pm$ 0.2	4.6 $\pm$ 0.2	7.2 $\pm$ 0.6
	Lactating	−D	8.8 $\pm$ 0.1‡	6.9 $\pm$ 0.3*‡	3.8 $\pm$ 0.2	6.7 $\pm$ 0.7
3	Non-lactating	−D	10.3 $\pm$ 0.1	10.1 $\pm$ 0.3	5.1 $\pm$ 0.2	4.6 $\pm$ 0.7
	Non-lactating	+D	10.5 $\pm$ 0.1	10.2 $\pm$ 0.3	4.7 $\pm$ 0.2	5.6 $\pm$ 0.9

The +D diet contained 5 IU D₃ per g diet and both +D and −D diets contained 0.4% Ca and 0.4% P. Non-lactating rats in experiment 2 were obtained as such from the supplier, while for experiment 3 rats were rendered non-lactating by removing their pups on the 8th day of lactation. The weight ranges for lactating and non-lactating rats were 240–390 g and 313–426 g, respectively. The mean weights for groups of rats within an experiment were not significantly different from each other ($P > 0.05$), neither were the weight gains by litters from −D and +D mothers. Each Ca and P value is the mean $\pm$ s.e. of 7–11 rats. Each $1\alpha,25-(\text{OH})_2\text{D}_3$ value is the mean $\pm$ s.e. of three or four pools of blood, each from three rats (except for lactating −D rats in experiment 1, two pools from five rats); determinations were in triplicate.

*Different from initial (as paired differences) and from lactating +D where appropriate, $P < 0.01$.

†Different from non-lactating, $P < 0.01$.

‡Different from non-lactating, $P < 0.001$.

§Different from lactating −D, $P < 0.025$.

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- 1 Toverud, S. U., Harper, C. & Munson, P. L. *Endocrinology* **99**, 371–378 (1976).
- 2 Toverud, S. U. *Transfer of Calcium and Strontium Across Biological Membranes* (ed. Wasserman, R. H.) 341–358 (Academic, New York, 1963).
- 3 Hughes, M. R., Brumbaugh, P. F., Haussler, M. R., Wergedal, J. E. & Baylink, D. J. *Science* **190**, 578–580 (1975).
- 4 Lawson, D. E. M., Fraser, D. R., Kodicek, E., Morris, H. R. & Williams, D. H. *Nature* **230**, 228–230 (1971).
- 5 Hughes, M. R., Baylink, D. J., Jones, P. G. & Haussler, M. R. *J. clin. Invest.* **58**, 61–70 (1976).
- 6 Hughes, M. R. *et al.* *Endocrinology* **100**, 799–806 (1977).
- 7 MacIntyre, I. *et al.* *Clin. Endocrinol.* **5**, Suppl. 85S–95S (1976).
- 8 Tanaka, Y. & DeLuca, H. F. *Archs Biochem. Biophys.* **154**, 566–574 (1973).
- 9 Fraser, D. R. & Kodicek, E. *Nature new Biol.* **241**, 163–166 (1973).
- 10 Garabedian, M., Holick, M. F., DeLuca, H. F. & Boyle, I. T. *Proc. natn. Acad. Sci. U.S.A.* **69**, 1673–1676 (1972).
- 11 Chen, P. S., Toribara, T. Y. & Warner, H. *Analyt. Chem.* **28**, 1756–1758 (1956).
- 12 Spanos, E. *et al.* *Life Sci.* **19**, 1751–1756 (1976).
- 13 Pike, J. W., Toverud, S. U., Boass, A., McCain, T. & Haussler, M. R. *Proc. 3rd Workshop Vitamin D*, Pacific Grove, California (1977).
- 14 Schneider, L. E., Haussler, M. R., Schedl, H. P. & McCain, T. A. *Clin. Res.* **24**, 567A (1976).
- 15 Spanos, E. *et al.* *Molec. cell. Endocrinol.* **5**, 163–167 (1976).
- 16 Toverud, S. U. *Acta physiol. scand.* **62** Suppl. 234, 1–31 (1964).
- 17 Shah, B. G. & Draper, H. H. *Am. J. Physiol.* **211**, 963–966 (1966).
- 18 Mainoya, J. R. *Endocrinology* **96**, 1165–1170 (1975).

and then grown as described by Nimrod and Lindner⁸. The following substances were added where indicated: hFSH (0.1 or 0.5 $\mu\text{g ml}^{-1}$, LER-1801-3); androstenedione (0.1 $\mu\text{g ml}^{-1}$); oestradiol-17 β (1.0 $\mu\text{g ml}^{-1}$); insulin (5.0 $\mu\text{g ml}^{-1}$, Squibb); and 8-bromo cyclic AMP (1.0 mM). When the culture period exceeded 48 h, the medium was changed with fresh addition of hormone. At the end of the culture period, the medium was removed and GC were collected using a rubber scraper (cell cultures) or collected from the ovarian segments as described above (organ cultures), and placed in buffered sucrose solution⁵. The cells were sedimented by centrifugation (500g for 8 min) and resuspended in a small volume of buffer by repeated passage through a Pasteur pipette. Highly purified hCG (13,600 U mg^{-1} , Serono, Italy) was labelled with ¹²⁵I (20–50 $\mu\text{Ci per } \mu\text{g hCG}$) by the lactoperoxidase method of Miyachi *et al.*¹⁰ and used in the assay of hCG binding⁵. Briefly, cell suspensions (50 μl containing 2×10^5 to 5×10^5 cells) were incubated for 3 h at 37 °C in a shaking incubator together with 50 μl of buffer containing 7–11 ng ml^{-1} ¹²⁵I-hCG with or without an excess of unlabelled hCG (Pregnyl, Organon; 0.5 U per sample). The difference between radioactivity bound to the cells in the presence and absence of unlabelled hormone was taken to represent specific binding. Progesterone and 20 α -dihydroprogesterone accumulation in the culture medium was determined by radioimmunoassay using specific antibodies¹¹.

Specific binding of ¹²⁵I-hCG to the cultured cells (48 h) was very low (0.16 fmol per 10^6 cells) even compared with that obtained with freshly collected cells (0.39 fmol per 10^6 cells). Culture of these cells with FSH, or with FSH in combination with androstenedione, oestradiol-17 β or insulin, had no effect on ¹²⁵I-hCG binding, although the cells clearly responded to FSH with an increased release of progestins into the medium and androstenedione augmented this effect as previously reported⁶.

By contrast, exposure of ovarian fragments in organ culture to FSH (0.1–0.5 $\mu\text{g ml}^{-1}$) for 48 h greatly augmented specific binding of ¹²⁵I-hCG to GC (1.19 $\pm$ 0.14 fmol per 10^6 cells, mean $\pm$ s.e.m., $n=20$, compared with 0.26 $\pm$ 0.03, $n=22$ in untreated controls, $P<0.001$ by Student's *t* test; compare Table 1). Extension of the culture with FSH to 96 h resulted in a further increase in the number of ¹²⁵I-hCG binding sites (Fig. 1). Addition of androstenedione (Table 1)

In vitro induction of binding sites for hCG in rat granulosa cells by FSH

THE granulosa cells (GC) of the Graafian follicle are considered to be the target of the cyclic surge of luteinising hormone (LH) which initiates ovulation and subsequent luteinisation of the follicle. It has been demonstrated that GC acquire receptors for LH during the final stages of follicular growth^{1,2}. In the immature hypophysectomised rat, treatment with oestrogen stimulates GC proliferation and growth of a large number of pre-antral follicles^{3,4}. Cells from these follicles are endowed with follicle-stimulating hormone (FSH) receptors⁵ and respond to FSH with increased progestin production⁶. They contain few or no LH receptors, however, as reflected by their negligible ability to bind ¹²⁵I-hCG (ref. 7) or to respond to LH with increased progestin production in culture (G. F. Erickson and A.N., unpublished). A dramatic increase of ¹²⁵I-hCG binding was demonstrated in GC following sequential treatment of immature hypophysectomised rats with oestradiol-17 β and FSH (refs 7, 8). To clarify the cellular mechanism of the induction of the LH receptors, we examined the induction of LH receptors by FSH *in vitro* and show that hCG binding sites can be induced by FSH in GC of pre-antral follicles in cultures of ovarian fragments, but not in isolated GC cultured as monolayers.

Wistar-derived immature hypophysectomised rats (28–30 d old) were each given four daily subcutaneous injections of 0.5 mg diethylstilboestrol in peanut oil (Hx-DES rats) and on day 5, the ovaries were excised aseptically. For organ culture, each ovary was sectioned into 8–12 fragments and these were placed on the grid of a Falcon organ culture dish and incubated⁹. For cell cultures, GC were isolated by puncture of follicles and gentle expression into the medium

Fig. 1 *In vitro* induction of hCG binding sites by FSH in GC during organ culture of ovaries from Hx-DES rats. Time course of increase in binding sites; $\blacktriangle$, FSH-treated (0.5 $\mu\text{g ml}^{-1}$); $\bullet$, no hormone supplementation. Vertical bars, $\pm$ s.e.m. ($n=3$).

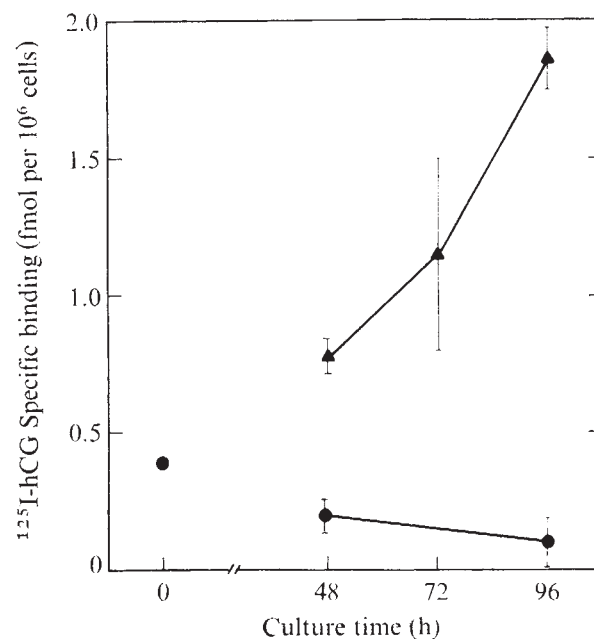


Table 1 The effect of FSH, androstenedione (Ad) and 8-bromo cyclic AMP on ^{125}I -hCG binding to the granulosa cells in organ cultures of ovaries from oestrogen-treated immature hypophysectomised rats.

Experiment no.	Culture conditions (48 h)	^{125}I -hCG binding (fmol per 10^6 cells)	n
1	No hormones	0.18 ± 0.03	5
	FSH ($0.1 \mu\text{g ml}^{-1}$)	1.23 ± 0.30	3
2	FSH + Ad ($1 \mu\text{g ml}^{-1}$)	1.03 ± 1.47	2
	No hormones	0.24 ± 0.05	5
3	FSH ($0.1 \mu\text{g ml}^{-1}$)	1.17 ± 0.46	5
	No hormones	0.45 ± 0.07	4
4	FSH ($0.5 \mu\text{g ml}^{-1}$)	1.15 ± 0.27	4
	No hormones	0.19 ± 0.04	4
5	FSH ($0.5 \mu\text{g ml}^{-1}$)	1.12 ± 0.29	4
	No hormones	0.26 ± 0.06	4
	FSH ($0.5 \mu\text{g ml}^{-1}$)	1.31 ± 0.23	4
	8-Bromo cyclic AMP (1.0 mM)	0.25 ± 0.08	4

Uptake by GC pooled from segments of two ovaries was determined in triplicate. Values shown are the mean $\pm$ s.e.m. of values obtained from n replicate cell pools or individual values where $n < 3$.

or oestradiol- 17β (data not shown) together with FSH did not modify the FSH effect. Culture of ovarian segments with 8-bromo cyclic AMP failed to induce hCG binding.

Organ cultures of ovarian fragments produced appreciable amounts of progestins (progesterone plus 20α -dihydroprogesterone, 140 ± 26 ng per dish per 48 h; mean $\pm$ s.d.), but in contrast to isolated GC cultures, failed to respond consistently to FSH with increased progestin production, whether or not androstenedione or oestradiol- 17β were also added. 8-Bromo cyclic AMP likewise failed to stimulate progestin production in the organ cultures.

The present study represents the first demonstration of an *in vitro* induction of LH receptors in ovarian cells. The failure of 9-bromo cyclic AMP to mimic this FSH effect could signify either that this particular response is not mediated by the adenylate cyclase system, or that penetration of the follicle cells by the nucleotide was inadequate. The low level of ^{125}I -hCG binding in GC of Hx-DES rats offers an advantage over GC from mature follicles in that it eliminates the problem of loss of initial receptor sites during culture. Previously published data on stimulation of hCG binding in porcine GC cultures by FSH (ref. 12) could be interpreted as a reflection of better maintenance of pre-formed receptors, rather than the induction of new binding sites in the presence of this hormone. We were unable to induce the appearance of new receptor sites for hCG by isolated GC after culture with FSH, although the isolated cells responded to FSH with increased progestin production. The difference in the behaviour of GC when cultured alone or in intact follicles suggests the participation of other cellular components of the ovary, or their products, in the induction of the LH receptor by FSH.

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¹ Channing, C. P. & Kammerman, S. *Endocrinology* **92**, 531–540 (1973).

² Lee, C. Y. *Endocrinology* **99**, 42–48 (1976).

³ Pencharz, R. I. *Science* **91**, 554–555 (1940).

⁴ Williams, P. C. J. *Endocr.* **44**, 127–136 (1945).

⁵ Nimrod, A., Erickson, G. F. & Ryan, K. J. *Endocrinology* **98**, 56–64 (1976).

⁶ Nimrod, A. & Lindner, H. R. *Molec. cell. Endocr.* **5**, 315–320 (1976).

⁷ Zeleznik, A. J., Midgley, A. R., Jr & Reichert, L. E., Jr *Endocrinology* **95**, 818–825 (1974).

⁸ Richards, J. S. et al. *Endocrinology* **99**, 1562–1570 (1976).

⁹ Tsafiri, A., Lindner, H. R., Zor, U. & Lamprecht, S. A. *J. Reprod. Fert.* **31**, 39–50 (1972).

¹⁰ Miyachi, Y., Vaitukaitis, J. L., Nieschlag, E. & Lipsett, M. P. *J. clin. Endocr. Metab.* **34**, 23–28 (1972).

¹¹ Bauminger, S., Kohen, F. & Lindner, H. R. *J. Steroid Biochem.* **5**, 739–747 (1974).

¹² Channing, C. P. *Proc. Soc. exp. Biol. Med.* **149**, 238–241 (1975).

Morphological and biochemical heterogeneity of cholinergic synaptic vesicles

WE present findings which seem to show that in an innervated perfused electric organ preparation from *Torpedo* the uptake of freshly synthesised acetylcholine by synaptic vesicles is linked to the discharge of previously stored transmitter by means of at least one cycle of exo- and endocytosis.

The electromotor synapse of *Torpedo* has provided a valuable model system for investigating the molecular basis of cholinergic transmitter storage and release¹. Although the numerous synaptic vesicles present in the nerve terminals seem to constitute a static and homogeneous population when the unstimulated tissue is viewed in the electron microscope, there is evidence that they are, in fact, morphologically and biochemically labile and heterogeneous. Thus, stimulation *in vivo* for short periods at 5 Hz caused marked vesicle depletion and reduction in size² and vesicles isolated from stimulated organs by submitting cytoplasmic extracts to density gradient centrifuging in a zonal rotor had less acetylcholine (ACh) and lower ACh/ATP ratios than those from unstimulated organs^{3,4}. On recovery from stimulation⁵, these changes are reversed, but vesicle numbers and diameters are more rapidly restored than vesicular ACh or ATP. The different types of vesicle do not differ sufficiently in physical properties to be sharply separated on a density gradient, though ACh/ATP ratios are observed to vary across the zonal vesicle peak⁴.

We aimed to modify the buoyant density of vesicles that had undergone exo- and endocytosis by incorporating into them dense material from the extracellular fluid. Sucrose, a constituent of a recently described *Torpedo* Ringer solution⁶, proved suitable for this purpose.

Innervated blocks of electric tissue 20–50 g in weight and comprising the territory of a single nerve were perfused with a physiological saline of osmolarity made equal to that of elasmobranch tissue by the inclusion of sucrose (200 mM) and urea (400 mM) (ref. 6). The blocks were cannulated via the blood vessels which accompany the nerves and supply the same territory. Blocks were stimulated via the nerve either moist, in air, or submerged in the perfusion medium; no significant differences were observed with the two methods. The response of the organ was monitored by electrodes placed on its ventral and dorsal surfaces. Whole tissue samples were taken for electron microscopy and vesicles were isolated from cytoplasmic extracts of the blocks on zonal density gradients essentially as described before^{2,5,8}.

To avoid vesicle depletion due to repetitive stimulation we used a low stimulation frequency (0.1 Hz) at which the response of the tissue blocks decayed to about 20–30% of the initial response (27 V) after 1,800 stimuli and the total tissue ACh fell less, to about 30–40% of its initial value (of $1,300 \text{ nmol g}^{-1} \pm 367 \text{ s.e.m.}$, $n=3$). Vesicle numbers (per μm^2 of presynaptic terminal cross section) were not reduced; rather there was a small (up to 50%) increase which might have been caused by volume changes in the terminals during perfusion. There was, however, the same progressive appearance in whole tissue sections of a second population of vesicles about 25% smaller in diameter than the initial population, as occurred at 5 Hz.

When dextran (molecular weight 10,000, 6–10%) was added to the perfusion fluid, few or no dextran particles

were taken up into the vesicles during an initial 3 h perfusion period. When stimulation at 0.1 Hz was applied, however, there was a progressive uptake, mainly into the population of small vesicles (Fig. 1a). After 1,800 impulses, 80% of all vesicles (four experiments) were found to belong to the small diameter population and of these 91% contained dextran. Thus, transmitter release is accompanied by vesicle exo- and endocytosis.

To determine whether vesicles undergoing cycles of exo- and endocytosis were capable of taking up transmitter, ^3H -acetate (sodium salt; $0.5\text{--}1.5\ \mu\text{Ci ml}^{-1}$, $5\ \mu\text{M}$), a precursor of tissue ACh (ref. 7), was added to the perfusion medium; the vesicles were isolated⁸ and vesicular ACh was extracted for assay and separated from unesterified acetate for

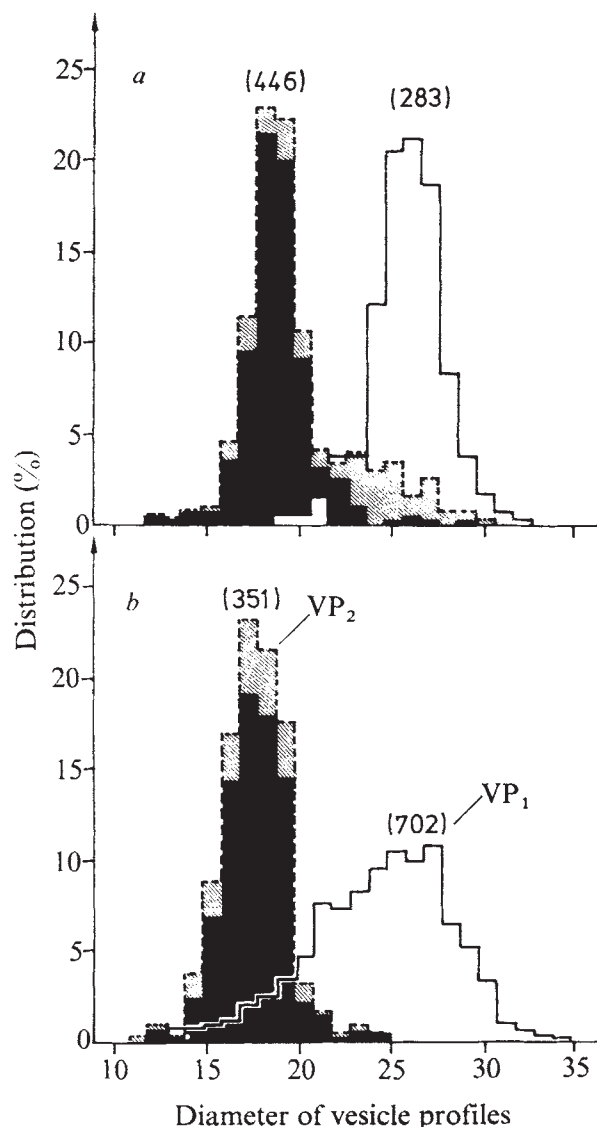


Fig. 1 *a*, Effect of stimulation (at 0.1 Hz) on synaptic vesicle profile diameters and the uptake of dextran into synaptic vesicles. The diagrams are normalised distributions of profile diameters in (continuous lines) unstimulated blocks and (dashed lines) blocks stimulated with 1800 pulses. Black blocks show the proportion of profiles of a given diameter that had a dextran particle in the lumen. Numbers in brackets give the number of vesicles measured. Only the small vesicles generated by stimulation contain dextran. *b*, Similar normalised distributions of vesicle profile diameters in vesicles isolated after 1,800 pulses. Note that fraction VP₁ (Fig. 2b) contains only the larger vesicle population whereas the smaller vesicles are recovered in VP₂. Electron-dense granules are restricted to the vesicles in VP₂ and the distribution of profile diameters in this vesicle fraction is similar to that of the newly formed small vesicle population shown in *a*. Abscissa: arbitrary scale.

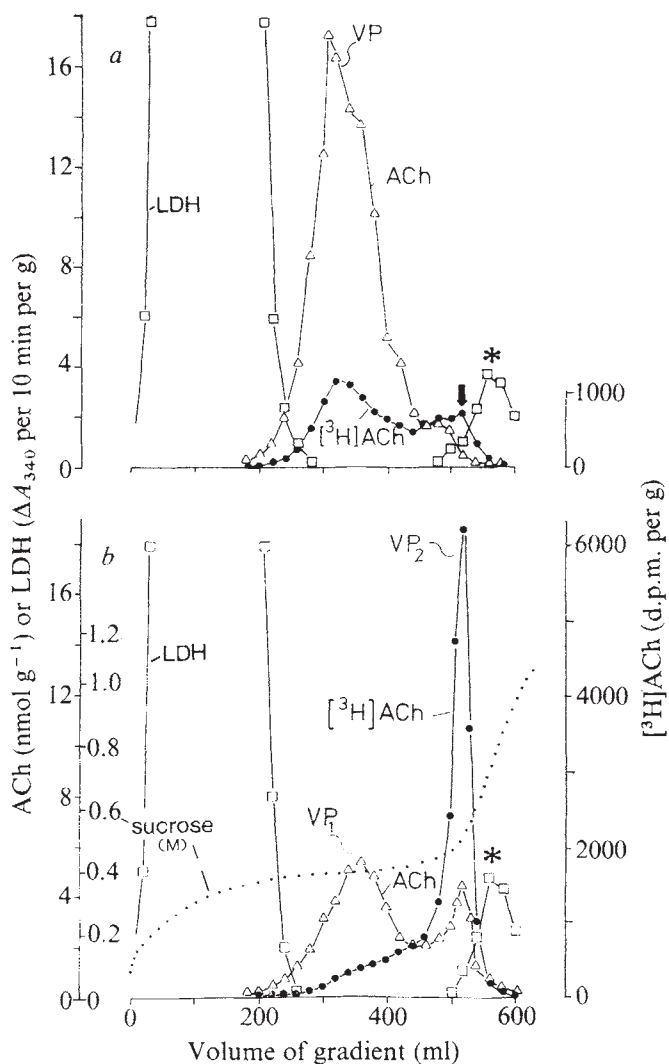


Fig. 2 *a*, Separation, in a zonal density gradient, of components of a cytoplasmic extract of an unstimulated tissue block showing peak (VP) of vesicular ACh, coincident peak and (arrow) shoulder of ^3H -ACh. *b*, Result of stimulation, showing two peaks of vesicular ACh, VP₁ corresponding to, but smaller than VP in (a) and VP₂ corresponding to the shoulder in (a) at the inflexion of the sucrose gradient. Peak VP₂ is highly labelled and does not coincide with the peak of entrapped cytoplasm indicated by lactate dehydrogenase (LDH) (*).

counting by liquid ion-exchange partition⁹. Whereas there was some incorporation of radioactivity into the single peak of endogenous vesicular ACh derived from unstimulated blocks (VP, Fig. 2a), stimulation caused the appearance, in a denser region of the gradient, of a second highly radioactive peak of ACh (VP₂, Fig. 2b) containing about 20% of the total vesicular ACh coincident in position with a shoulder of radioactive ACh observed with the material from control blocks (arrow, Fig. 2a). Meanwhile the height of the peak (VP₁) corresponding to VP was reduced.

The specific radioactivity of the ACh of VP₂ was 16.6 ± 4.8 s.e.m. ($n=5$) times greater than that of VP₁ and 9.2 ± 2.4 ($n=4$) times than that of the parent cytoplasmic extract. Furthermore the ACh of the parent cytoplasmic extract had a specific radioactivity twice that of the tissue ACh (1.9 ± 0.1 , $n=7$). Similar results have been obtained with radioactive choline (J.Susziw and H.Z., unpublished) and the incorporation is blocked by hemicholinium-3.

Since the region of the gradient occupied by VP₂ overlaps the rising portion of a peak of membrane fragments, the possibility exists that VP₂ consists of cytoplasmic ACh sequestered within membrane bound particles. That this is

not so was shown by its lack of coincidence with small peaks of the cytoplasmic markers lactate dehydrogenase (Fig. 2b) and choline acetyltransferase, and with a peak of separately prepared synaptosomes ('T-sacs')¹⁰ labelled with ¹⁴C-ACh and added to the cytoplasmic extract from stimulated tissue blocks (Fig. 3). There is good reason to assign the ACh of VP₂ to the population of small vesicles found mixed with membrane fragments in this region of the gradient. When dextran as well as ³H-acetate was perfused through stimulated tissue blocks, those vesicles could be readily distinguished electron microscopically from the larger membrane fragments by their content in electron-dense particles and Fig. 1b demonstrates that the diameter distribution of VP₁ and VP₂ is essentially the same as that between the large and small vesicle populations of intact terminals (Fig. 1a). Furthermore, electron-dense granules can be found only in the small vesicle population. The presence of dextran in the perfusion medium is not, however, essential for the separation of the two populations of vesicles; the essential component is sucrose.

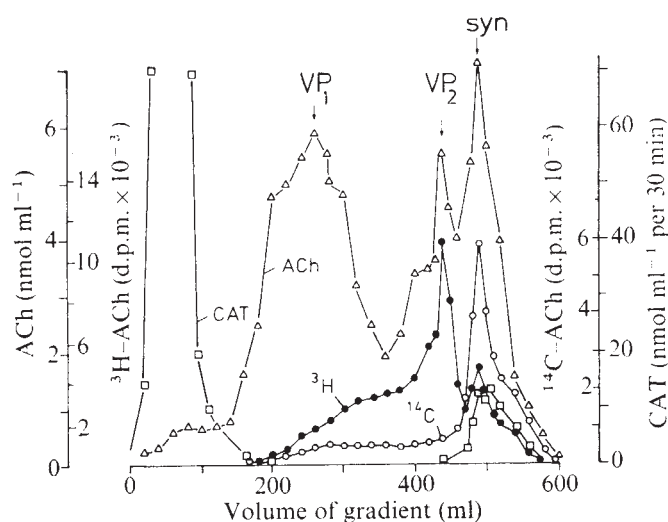


Fig. 3 Separation, in a zonal density gradient, of components of a cytoplasmic extract of a stimulated block to which a preparation of synaptosomes ('T-sacs') labelled with ¹⁴C-ACh, had been added. Note that VP₂ is well separated from the synaptosome peak (Syn) identified by its content of ¹⁴C-ACh and choline acetyltransferase (CAT).

These results seem to show (1) that *Torpedo* electro-motor synaptic vesicles, like brain cortex vesicles¹¹, are metabolically heterogeneous with respect to ACh uptake. (2) That the subpopulation of vesicles taking up newly synthesised ACh has undergone at least one cycle of exo-/endocytosis. And (3), that such vesicles can be separated from the main population by an artificially induced alteration of density, a technique similar to that used¹² to separate liver lysosomes from mitochondria. The implications of these findings for the vesicle theory of transmitter storage and release¹³ are now being examined.

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- 1 Whittaker, V. P. & Zimmermann, H. in *Biochemical and Biophysical Perspectives in Marine Biology* (eds Malins, D. C. & Sargent, J. R.) 67-116 (Academic, London, 1976).
- 2 Zimmermann, H. & Whittaker, V. P. *J. Neurochem.* **22**, 435-450 (1974).
- 3 Dowdall, M. J., Boyne, A. F. & Whittaker, V. P. *Biochem. J.* **140**, 1-12 (1974).
- 4 Dowdall, M. J. & Zimmermann, H. *Brain Res.* **71**, 160-166 (1974).
- 5 Zimmermann, H. & Whittaker, V. P. *J. Neurochem.* **22**, 1109-1114 (1974).
- 6 Babel-Guérin, E. *J. Neurochem.* **23**, 525-532 (1974).
- 7 Israël, M. & Tuček, S. *J. Neurochem.* **22**, 487-493 (1974).
- 8 Whittaker, V. P., Essmann, W. B. & Dowe, G. H. C. *Biochem. J.* **128**, 833-846 (1972).
- 9 Fonnum, F. *Biochem. J.* **115**, 465-472 (1969).
- 10 Dowdall, M. J. & Zimmermann, H. *Expl. Brain Res.* **24**, 8-9 (1975).
- 11 Barker, L. A., Dowdall, M. J. & Whittaker, V. P. *Biochem. J.* **130**, 1063-1080 (1972).
- 12 Thines-Sempoux, D. *Biochem. J.* **105**, 20P (1967).
- 13 MacIntosh, F. C. & Collier, B. *Hdb. exp. Pharmacol.* **42**, 99-228 (1976).

Interference by bromodeoxyuridine with differentiation in a prokaryote

ENDOSPORE formation in bacteria can be regarded as a primitive form of cellular differentiation as the mature spore has many features, both structural and biochemical, which distinguish it clearly from the growing cell. The extent to which bacterial sporulation can be used as a simple model for development in higher organisms depends on the similarity or otherwise of the control processes which influence cellular differentiation in both cases. It would thus seem important to draw as close an analogy as possible between prokaryotic and eukaryotic differentiating systems. With this in mind, the effect of 5-bromo-2'-deoxyuridine (BUdR) on growth and sporulation in *Bacillus subtilis* has been investigated. When incorporated into the DNA of eukaryotic cells, BUdR (an analogue of thymidine, TdR) selectively affects processes associated with differentiated and embryogenesis, without significantly altering cell growth or gross RNA and protein synthesis¹⁻³. The effects seem best explained by an alteration in transcription^{2,4,5}, possibly caused by tighter binding of chromosomal proteins^{6,7}. This may also be true for prokaryotes, because purified *lac* repressor and catabolite gene activator protein from *E. coli* bind more tightly to BUdR-substituted DNA⁸. Sporulation in *B. subtilis* is controlled to a considerable extent by sequential activation of genes, many of which remain repressed during vegetative growth (for review see ref. 9). We report here that BUdR, at a concentration which leaves growth unaffected, markedly reduces the capacity of *B. subtilis* to sporulate. This effect is comparable with the known interference of this analogue with cellular differentiation in eukaryotes.

B. subtilis 168 (*thy*⁻*A*, *trpC2*) was used as the parent strain, but even partial replacement of the necessary TdR in the medium by BUdR inhibited growth. To overcome this problem a mutant was isolated which still retained the original *thy*⁻*A* mutation, but was much less susceptible to the toxic effects of BUdR. This BUdR-tolerant strain (BUt-32) was able to grow normally (doubling time 36 min) in a rich casein hydrolysate (CH) medium¹⁰ with a BUdR:TdR ratio of 7.5 $\mu\text{g ml}^{-1}$:1.0 $\mu\text{g ml}^{-1}$. At higher ratios the growth rate was progressively affected. BUt-32 was selected by a procedure similar to that described for the isolation of 5-bromouracil (BU)-tolerant mutants of *B. subtilis*¹¹. Like the BU-tolerant strains, BUt-32 was unable to grow for more than a few generations with BUdR alone, and a small amount of the natural nucleoside had to be present during growth and sporulation. The basis of the increased resistance to analogue toxicity has not been investigated in either case.

The buoyant density in CsCl of DNA prepared from BUt-32 cells grown with TdR alone was compared with that from cells grown with BUdR and TdR (7.5:1.0). The degree of BUdR substitution can be estimated from the difference in buoyant density between poly d(A-T) and poly d(A-BU) which is 200 mg ml^{-1} (ref. 12). Because *B. subtilis* 168 DNA contains only 28% TdR residues¹³ compared with 50% in poly d(A-T), complete BUdR substitution should give a density increase of about 112 mg ml^{-1} . The density difference found averaged 35 mg ml^{-1} and in separate experiments varied between 40 and 28 mg ml^{-1} . This represents an average 30% substitution by BUdR, a value compatible with earlier results which showed a marked preferential

Table 1 Occurrence of sporulation events in cells supplemented with TdR plus BUdR or TdR alone

Growth and resuspension of cells in media containing*	Exoprotease† (U ml ⁻¹ of culture)	Alkaline phosphatase† (U ml ⁻¹ of culture)	Viable count‡ (colonies per ml × 10 ⁻⁵)	Heat resistance‡ (colonies per ml × 10 ⁻⁵)
TdR (8.5 µg ml ⁻¹)	0.08	0.21	4,030	818
TdR (1 µg ml ⁻¹) + BUdR (7.5 µg ml ⁻¹)	0.14	0.25	3,590	9

*Cells of BUt-32 growing exponentially in rich CH medium were resuspended in a poor minimal salts medium to initiate sporulation¹⁰. Samples of the cultures were assayed at hourly intervals for exoprotease activity¹⁵ and alkaline phosphatase activity¹⁶. Viable count was determined by dilution and plating on nutrient agar. Heat-resistance was determined in the same way, after heating a cell sample at 85 °C for 15 min. The values shown are the results of a typical experiment.

†Maximum values obtained, 4 h and 6 h after resuspension for exoprotease and alkaline phosphatase respectively. 1 unit of enzyme activity is defined as a change in absorption of 0.1 min⁻¹ at 595 nm and 410 nm for exoprotease and alkaline phosphatase respectively.

‡Measured 8 h after resuspension of cells.

uptake and incorporation by *B. subtilis* of the natural nucleoside, TdR, over BUdR^{11,14}.

The effect of this degree of substitution on the ability of the cells to sporulate was investigated (Table 1). During sporulation two early biochemical marker events, exoprotease (Pr) activity and alkaline phosphatase (AP) activity, were assayed and heat-resistance was used as a measure of the number of mature spores formed. The presence of BUdR had the effect of markedly increasing the activities of both Pr and AP over the control values, but the final yield of mature spores was reduced to about 1% of that of the control culture. The low degree of sporulation was not due to loss of viability by the cells incorporating BUdR (Table 1) or to delayed sporulation, for prolonged incubation (24 h) failed to increase the percentage spore yield relative to the control. Prevention of DNA replication is known to prevent sporulation^{17,18}, but DNA synthesis, measured by uptake of ¹⁴C-TdR into acid-insoluble material, continued in a similar manner in both cultures for 2 h after initiation to sporulation (results not shown).

Examination of cells by electron microscopy 7 h after initiation, when the bulk of the control cells had formed readily identifiable spores, showed that BUdR incorporation prevented most cells from reaching the stage of spore septum formation (stage II). Most of the cells incorporating BUdR were normal in length and had nuclear material in the form of an axial filament. Rarely, cells were seen with wall protrusions or 'spikes' at one pole (a recognised stage preceding spore septum formation¹⁹), but generally the cells had no characteristics of sporulating cells. The occasional elongated cell or multiseptate cell was also observed.

This apparent interference by BUdR with an early stage of sporulation was confirmed by exposure of cells grown with TdR alone to BUdR at intervals after initiation to sporulation (Table 2). Cells initiated in the presence of the analogue had a markedly reduced capacity to form spores, only slightly better than that exhibited by cells grown as well as sporulated with BUdR. Initiation of cells in resuspension medium with TdR alone followed by transfer to resuspension medium containing BUdR 1 h after initiation gave a much increased spore yield. By 2 h after initiation in TdR medium exposure of cells to BUdR had essentially no effect on subsequent sporulation. Conversely, cells grown with BUdR, but initiated to sporulation in its absence exhibited a much increased capacity to sporulate, roughly about half that of control cells previously grown with TdR alone (Table 2). This indicated that BUdR incorporation during growth had little subsequent effect on sporulation if initiation occurred in the absence of the analogue. Initiation in the presence of BUdR followed by transfer of cells to a medium containing TdR alone 1, 2 or 3 h later produced a much reduced spore yield when assayed at 8 h, but by 24 h the level of sporulation had recovered almost to that found in control cells. This recovery indicated that the inhibitory effect of BUdR on

sporulation was reversible on removal of the analogue followed by continued incubation in the presence of TdR alone.

Sporulation was induced in these experiments in shift-down conditions involving transfer of cells from a rich to a poor medium. This would have necessitated the general derepression of many genes involved in biosynthetic processes. If BUdR incorporation was affecting transcription generally, the effect on sporulation might be caused, for example, by lack of transcription for a required amino acid rather than an effect on sporulation itself. This explanation seems unlikely for two reasons. First, BUt-32 grew normally (doubling time 65 min) in the minimal resuspension medium supplemented with glucose (0.2%) with a BUdR:TdR ratio of 7.5 µg ml⁻¹:1.0 µg ml⁻¹. After 24 h only the occasional spore was detected in this medium, yet more than 50% of the cells in a culture grown in the same medium with TdR alone sporulated. Second, when L-histidine (500 µg ml⁻¹) was included in the normal resuspension medium in an experiment similar to that described in Table 1, the rate of synthesis of the inducible enzyme histidase was the same whether the cells had incorporated BUdR or not.

The precise nature of the sporulation-associated events affected by BUdR incorporation is unknown, but certain

Table 2 Effect on spore yield of removal or addition of BUdR at intervals after initiation of cells to sporulation

Time of removal of cell sample after resuspension* (h)	Spore yield†			
	Removal from TdR + BUdR medium		Removal from TdR + BUdR to TdR medium	
	8 h	24 h	8 h	24 h
0	37	87	280	390
1	310	630	24	420
2	580	1020	13	380
3	710	1040	16	420

*Parallel cultures (50 ml) of BUt-32 were grown in CH medium in the presence of either TdR (1 µg ml⁻¹) + BUdR (7.5 µg ml⁻¹) or TdR (8.5 µg ml⁻¹). To initiate sporulation 10-ml and 40-ml portions of each culture were centrifuged and resuspended in minimal salts medium. For cells grown in the presence of TdR alone the 40-ml portion was resuspended with TdR (8.5 µg ml⁻¹) and the 10-ml portion with TdR (1 µg ml⁻¹) + BUdR (7.5 µg ml⁻¹); the latter represented a time zero transfer to the new medium. Cells grown with TdR + BUdR were treated in a similar manner except that the 40 ml portion was resuspended with TdR + BUdR and the 10 ml portion with TdR only. At hourly intervals for the next 3 h further 10-ml samples were removed from each 40 ml culture, the cells collected on a membrane filter and then resuspended in 10 ml pre-warmed salts medium such that cells previously exposed to TdR alone were introduced into TdR + BUdR medium and vice versa.

†Heat-resistant colonies per ml × 10⁻⁵ determined 8 h and 24 h after resuspension as described in the legend to Table 1. Values for control cultures were 620 (8 h) and 1,130 (24 h) for cells grown and sporulated in the presence of TdR alone and 14 (8 h) and 41 (24 h) for cells grown and sporulated in the presence of TdR + BUdR.

observations deserve comment. The increase in AP activity during sporulation is normally associated with stage II (ref. 21), but BUdR incorporation allowed the increase in activity without the accompanying morphological changes. In addition, both Pr and AP activities were markedly increased over control values. This overproduction of enzyme activity is reminiscent of that seen in certain *spo* mutants⁹ and has also been observed during BUdR incorporation in eukaryotes (noted in refs 7 and 8). The inhibitory effect of BUdR on sporulation only occurred within an initial 2 h after initiation, when DNA replication was in progress, and the effect was reversible on removal of the analogue. Sensitivity to BUdR during a restricted interval of development and reversibility of its effects by further rounds of DNA replication in its absence have been observed in many eukaryotic systems^{1,2}. The presence of BUdR during the growth phase of the cells before the induction of sporulation had only a marginal effect on their subsequent sporulation capacity. This raised the possibility that BUdR inhibition of sporulation was primarily a secondary effect not related to its incorporation into DNA. For example, effects on the cytoplasmic membrane might be expected to interfere more with sporulation than with growth²⁰. BUdR had no effect on the sporulation capacity of wild-type *B. subtilis*, however, which suggests that a simple phenotypic effect of this sort is unlikely. At present it must be assumed that the inhibitory effect of BUdR is a consequence, for the most part, of its incorporation into DNA during chromosome replication at the onset of sporulation. This incorporation might interfere with the regulation of the process in some way, perhaps by causing an alteration in transcription due to tighter binding of regulatory proteins or by an alteration in the stability of chromosome-membrane complexes as has been suggested for other systems^{1,2,20}.

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- ¹ Holtzer, H. *et al. Curr. Top. dev. Biol.* **7**, 229-256 (1972).
- ² Rutter, W. J., Pictet, R. L. & Morris, P. W. A. *Rev. Biochem.* **42**, 601-646 (1973).
- ³ Guespin-Michel, J. F. *et al. Expl Cell Res.* **98**, 184-190 (1976).
- ⁴ Hill, B. T., Tsuboi, A. & Baserga, R. *Proc. natn. Acad. Sci. U.S.A.* **71**, 455-459 (1974).
- ⁵ Jones, T. C. & Dove, W. F. *J. molec. Biol.* **64**, 409-416 (1972).
- ⁶ Lapeyre, J. N. & Bekhor, I. *J. molec. Biol.* **89**, 137-162 (1974).
- ⁷ Lin, S.-Y., Lin, D. & Riggs, A. D. *Nucleic Acids Res.* **3**, 2183-2191 (1976).
- ⁸ Lin, S.-Y. & Riggs, A. D. *Biochim. biophys. Acta* **432**, 185-191 (1976).
- ⁹ Piggot, P. J. & Coote, J. G. *Bact. Rev.* **40**, 908-962 (1976).
- ¹⁰ Sterlini, J. M. & Mandelstam, J. *Biochem. J.* **113**, 29-37 (1969).
- ¹¹ Bishop, R. J. & Sueoka, N. *J. Bact.* **112**, 870-876 (1972).
- ¹² Wake, R. G. & Baldwin, R. C. *J. molec. Biol.* **5**, 201-216 (1962).
- ¹³ Schildkraut, C. L., Marmur, J. & Doty, P. *J. molec. Biol.* **4**, 430-443 (1962).
- ¹⁴ Laird, C. D. & Bodmer, W. F. *J. Bact.* **94**, 1277-1278 (1967).
- ¹⁵ Dancer, B. & Mandelstam, J. *J. Bact.* **121**, 406-410 (1975).
- ¹⁶ Coote, J. G. *J. Bact.* **120**, 1102-1108 (1974).
- ¹⁷ Mandelstam, J., Sterlini, J. M. & Kay, D. *Biochem. J.* **125**, 635-641 (1971).
- ¹⁸ Dawes, I. W., Kay, D. & Mandelstam, J. *Nature* **230**, 635-641 (1971).
- ¹⁹ Yamamoto, T. & Balassa, G. *Molec. gen. Genet.* **106**, 1-13 (1969).
- ²⁰ Bohin, J. P., Rigomier, D. & Schaeffer, P. *J. Bact.* **127**, 934-940 (1976).
- ²¹ Glenn, A. R. & Coote, J. G. *Biochem. J.* **152**, 85-89 (1975).

Transcription of a mammalian sequence under phage λ control

RECOMBINANT DNA techniques have recently enabled bacterial plasmids carrying eukaryotic sequences synthesised *in vitro* from purified mRNAs to be constructed and cloned in *Escherichia coli*¹⁻¹⁰. We show here that a rabbit globin sequence is not transcribed in *E. coli* when carried by the pCR1 plasmid. However, after transfer into phage λ , the globin sequence is transcribed under λ control. Thus, there is no basic hindrance to the transcription of a specific mammalian sequence in *E. coli*.

Recombinant plasmids containing globin cDNA obtained as previously reported by us¹, carried only

a relatively short part of the globin sequence. For the construction of another series of plasmids⁷, much longer double-stranded globin DNA molecules were synthesised from cDNA copies of purified globin mRNA with reverse transcriptase in a 'self-priming' reaction⁸. A similar procedure for the synthesis of double-stranded DNA from cDNA has been independently devised by others⁶⁻⁹. Plasmids were thus obtained with the α or the β globin gene from rabbit⁷ and mouse¹⁰.

The protocol used for these experiments^{1,7,10} involved the reconstruction of an *EcoRI* site on each side of the integrated sequence. Analysis of many hybrid plasmids revealed that these two *EcoRI* sites were present only rarely, but were present, however, on one of the rabbit plasmids, pCR1 β G19, which carries the entire β globin sequence. Since there is also an *EcoRI* site within this sequence, digestion of pCR1 β G19 DNA yields, in addition to the parent plasmid DNA, two smaller fragments, A and B, 410 and 210 base pairs long respectively, which correspond to the 5' (A) and 3' (B) ends of globin mRNA⁷.

To determine whether the globin sequence is transcribed in *E. coli* harbouring pCR1 β G19, unlabelled RNA was extracted from exponentially growing cells, hybridised with ³H-globin cDNA, and the hybrids monitored with nuclease S1. There was little or no detectable globin RNA in conditions which we estimate would allow the detection of less than one molecule of globin RNA per bacterial cell (Table 3, line 1). We then explored the possibility that the rabbit sequence could be transcribed in *E. coli* when carried by another vehicle such as phage λ .

pCR1 β G19 DNA was digested to completion with *EcoRI*, and the fragments were recombined with the DNA of

Table 1 Characterisation of lysogens made from recombinant phages

DNA extracted from	³ H-globin DNA hybridised (c.p.m.)	Hybridisation ratio long-short/short	Recombinant structure
C600	8	—	—
C600 (pCR1 β G19)	663	0.17	pCR1-A-B
C600 (λ 1)	564	0.17	λ B
C600 (λ 2)	601	0.53	λ A
C600 (λ 3)	578	0.47	λ A
C600 (λ 4)	601	0.50	λ A
C600 (λ 5)	525	0.11	λ B
C600 (λ 6)	547	0.19	λ B
C600 (λ 7)	636	0.58	λ A
C600 (λ 8)	558	0.11	λ B
C600 (λ 11)	557	0.20	λ B
C600 (λ 12)	552	0.15	λ B
C600 (λ 13)	562	0.15	λ B
C600 (λ 14)	11	—	—
C600 (λ 15)	637	0.09	λ B
C600 (λ 16)	623	0.49	λ A
NoDNA	9	—	—

pCR1 β G19 DNA was purified and digested by *EcoRI* as described⁷. It was next recombined, in a 3:1 molar ratio with *EcoRI* digested DNA of phage 589, in the presence of T4 DNA ligase at 4 °C (refs 11, 18). The calcium method (slightly modified, as described in refs 11 and 18) was used for transfection in C600_{r_k⁻m_k⁻}. Infective centres were plated in the presence of W3110 *polA*⁻ in a 1:1 ratio with C600_{r_k⁻m_k⁻}. On the mixed lawn, *red*⁻ phages form small turbid plaques at 37 °C, which are easily scored by stabbing on to C600 and W3110 *polA*⁻ (ref. 11). 14 *red*⁻ phages (the putative recombinants) were re-isolated twice; stocks were made, and lysogens were isolated from a lawn of C600 spotted at 32 °C with about 10⁸ phages. After purification, the lysogens were grown at 32 °C in broth. The DNA of the 14 independent lysogens was then extracted from 5 ml of culture, immobilised on nitrocellulose filters and hybridised with 1,000 c.p.m. of ³H-globin cDNA (7 × 10⁶ c.p.m. μ g⁻¹, prepared as in ref. 8), for 18 h at 37 °C in 2 × SSC-50% formamide pH 7.2; the reaction volume was 4 μ l (refs 1, 14, 15). Note that the ³H-cDNA, made from a mixture of α and β globin RNAs, consists of about 2/3 of β chain DNA. The table also shows the ratio (long-short)/short of c.p.m. hybridised with long or short cDNA fractionated in a sucrose gradient as described in the text.

Table 2 Transcription of the globin sequence assayed by filter hybridisation

³ H RNA extracted from	Input (c.p.m.)	λ DNA	c.p.m. hybridised to				Globin-specific c.p.m.	Globin/λ
			pCR1βG19	pCR1	no DNA			
W3350 (λCI857)								
<i>cro</i> -P ⁻ <i>int</i> ⁻ 42 °C	228,000	50,440	150	185	120	—		
C600 (λ2) 32 °C	475,000	180	80	65	20	—		
42 °C	610,000	5,840	280	90	30	190	3.2%	
C600 (λ3) 32 °C	945,000	160	95	110	30	—		
42 °C	382,000	4,765	400	125	35	275	5.7%	
C600 (λ4) 32 °C	1,400,000	210	80	135	40	—		
42 °C	431,000	3,810	170	70	20	100	2.6%	
C600 (λ7) 32 °C	300,000	190	110	110	40	—		
42 °C	196,000	4,000	165	80	30	85	2.1%	

Four lysogens were grown in 63 medium supplemented with glucose, casaminoacids and vitamin B1 (ref. 16), for pulse labelling of 10 ml of culture with ³H-uridine (CEA, 25 Ci mM⁻¹, 20 μCi ml⁻¹). The RNAs were extracted as before¹⁶ and hybridised at 37 °C for 4 h, in a final volume of 4 μl of 2 × SSC–50% formamide, to filters carrying 1 μg of λ, pCR1βG19 or pCR1 DNA, or no DNA. Filters were processed as usual⁹. There is no cross hybridisation between the phage, plasmid and globin sequences as shown in the first line of the table, where λRNA from a overproducing strain¹⁸ was labelled and hybridised in similar conditions.

phage 589 in our collection. This phage was derived from λplac5 by a series of *in vivo* and *in vitro* manipulations (P.K. and D.G., in preparation). It has lost the *EcoRI* *lac* fragment (a 14% deletion) and has only one *EcoRI* sensitive site located within the *red* gene of λ (ref. 12). Integration of foreign DNA at this site makes the phage *red*⁻ so that recombinants are easily scored as small plaque formers on a mixed lawn (1:1 of *polA*⁺ and *polA*⁻ bacteria (ref. 12 and P.K. and D.G. in preparation). Recombinants of phage 589 (which carries the *cI857* mutation) lysogenise easily at 32 °C, at least at high multiplicity, in spite of the partial cold-sensitive character of such phages (F. Bregegere, J. Leal and P.K., in preparation). Since pCR1 DNA is too large to be accommodated within phage 589, the only recombinants which could be obtained are those carrying the A or B globin fragment, or both.

Recombinants were isolated, and a lysogen made with ³H-globin cDNA (ref. 1). Of 14 strains examined, 13 carried the lysogens, denatured, and immobilised on nitrocellulose

filters for hybridisation in formamide¹³ under paraffin oil¹⁴ with ³H-globin cDNA¹. Of 14 strains examined, 13 carried a globin sequence. Hybridisation was further carried out with cDNA fractionated in a sucrose gradient into short cDNA (< 250 nucleotides) or long cDNA (> 250 nucleotides) and with ³H-pCR1βG1 DNA. This allowed the identification of phages carrying the A or the B fragment, or both. In five strains out of thirteen, hybridisation with short cDNA was significantly reduced. These strains, therefore, are of the λ-A type, while the others are λ-B, or λ-A-B. Monitoring with ³H-pCR1βG1 DNA, a plasmid which carries only a short part of the globin sequence¹, indicated preferential hybridisation with DNA from the λ-A strains. This suggests that the portion of the globin sequence in pCR1βG1 is mostly in the A region, and that (with two possible exceptions) the eight other strains are λ-B rather than λ-A-B.

Is the globin sequence transcribed in non-induced or induced lysogens, and if so, is it transcribed from the correct or the anti-sense strand?

Attention was first focused on four λ-A strains. We took advantage of the availability of the globin sequence in two unrelated molecular vehicles (pCR1 and λ) (which allow it to be produced in large amounts) to assay transcription from λ-A by hybridisation to a large excess of pCR1βG19 DNA. Four λ-A lysogens were pulse labelled with ³H-uridine at 32 °C (3 min) or at 42 °C (2 min after an incubation of 3 min at 42 °C; early transcription in the *red* region is known to be maximal at about this time¹⁵). The radioactive RNAs were extracted and hybridised to excess pCR1βG19 and pCR1 DNA on filters. In all cases transcription of the globin sequence occurred in the induced, but not in the non-induced lysogens (Table 2). The rough assumption can be made that about 70% of the radioactivity hybridised to λ DNA reflects the *l* strand transcription of about 16% of the λ chromosome, that is, 7,000 base pairs. The transcription of 410 base pairs of globin sequence should correspond to about 3.5% that of λ, in relatively good agreement with figures of 2 to 5% derived from data in Table 2.

To determine whether the coding strand is transcribed in at least some of the strains, unlabelled RNA was extracted from non-induced lysogens and lysogens induced for 7 min. The unlabelled RNAs were hybridised with ³H-cDNA. On treatment with nuclease S1, protection of the ³H-cDNA is observed in two strains out of four, demonstrating that, in these two strains, the coding strand is transcribed (Table 2). When similar experiments were carried out with the other strains, transcription of the coding strand was observed in a total of eight strains out of the thirteen examined.

On methodological grounds, the above results illustrate the use of two unrelated vectors (λ and pCR1) carrying a

Table 3 Transcription of the globin sequence assayed by hybridisation to ³H-β globin cDNA.

RNA extracted from C600 harbouring	+S1	–S1	% resistance
pCR1βG19	63	296	12
λ1 I	172	341	45
λ2 I	73	318	14
λ3 {NI	26	332	0
I	30	346	0
λ4 {NI	27	357	0
I	198	335	55
λ5 I	48	305	3
λ6 {NI	49	274	3
I	169	296	53
λ7 {NI	24	311	0
I	208	344	60
λ8 I	43	277	1
λ11 I	153	296	45
λ12 I	54	255	5
λ13 I	174	275	57
λ14 I	49	273	3
λ15 I	219	262	81
λ16 I	220	330	63

200 ml of C600 *r_k*⁻ *m_k*⁻ *recBC*⁻ (pCR1βG19) were grown (in broth at 37 °C) as well as 200 ml of the various lysogens (grown in broth at 32 °C and induced at 42 °C for 7 min) and in some cases, 200 ml of the non-induced lysogens. Cells were collected at a density of 4 × 10⁸ bacteria per ml. The RNAs were extracted as before^{16,18} and concentrated to about 1 mg ml⁻¹. 100 μl were hybridised for 16 h at 70 °C with 700 c.p.m. of ³H-globin cDNA copied from the β chain (7 × 10⁶ c.p.m. μg⁻¹ as before) in a final volume of 120 μl in 2 × SSC. After incubation, the samples were divided into two, and precipitated with TCA after previous or no treatment with nuclease S1. A background of 30 c.p.m. was subtracted before calculating the percentage resistance.

I, induced; NI, non-induced.

similar DNA piece as a general tool for transcriptional studies (DNA from one recombinant can serve to monitor transcription from the other). Our results also show that *Eco*RI sites constructed *in vitro* have the expected properties for both endonuclease attack⁷ and *in vitro* recombination. In this process, the four more likely combinations (integration of fragment A, or B, in either direction) were observed suggesting that recombination occurred at random.

We show that a specific mammalian sequence can be transcribed from the coding strand in *E. coli* K12 under phage λ control. This is not unexpected in view of the known properties of the phage transcriptional systems¹⁶. The anti-termination properties of the *N* gene product make it likely that a number of sequences, including eukaryotic ones, can be transcribed under λ control, once integrated in appropriate phage early regions. This work provides an example of this, as a necessary step on the road to eukaryotic gene expression in *E. coli*.

Biohazards associated with the experiments described in this publication have been examined previously by the French National Control Committee. The experiments were carried out accordingly in P2-like conditions (in the NIH nomenclature). This work was supported by grants from CNRS, INSERM and DGRST in Paris, and by the FNRS in Geneva. One of us (F.R.) was supported by a short-term EMBO fellowship.

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- 1 Rougeon, F., Kourilsky, P. & Mach, B. *Nuclear Acids Res.* 2, 2365–2378 (1975).
- 2 Covey, C., Richardson, D. & Carbon, J. *Molec. gen. Genet.* 145, 155–158 (1976).
- 3 Jackson, D. A., Symons, R. M. & Berg, P. *Proc. natn. Acad. Sci. U.S.A.* 69, 2904–2909 (1972).
- 4 Rabbitts, T. H. *Nature*, 260, 221–225 (1976).
- 5 Maniatis, T., Kee, S. G., Efstratiadis, A. & Kafatos, F. C. *Cell* 8, 163–182 (1976).
- 6 Higuchi, R., Paddock, G. V., Wall, R. & Salser, W. *Proc. natn. Acad. Sci. U.S.A.* 73, 3146–3150 (1976).
- 7 Rougeon, F. & Mach, B. *J. biol. Chem.* 252, 2209–2217 (1977).
- 8 Rougeon, F. & Mach, B. *Proc. natn. Acad. Sci. U.S.A.* 73, 3148–3422 (1976).
- 9 Efstratiadis, A., Kafatos, F. C., Maxam, A. M. & Maniatis, T. *Cell* 7, 279–288 (1976).
- 10 Rougeon, F. & Mach, B. *Gene* (in the press).
- 11 Salser, W. *et al. Fedn Proc.* 35, 23–35 (1975).
- 12 Murray, N. E. & Murray, K. *Nature*, 251, 476–481 (1974).
- 13 Kourilsky, P., Leidner, J. & Tremblay, G. Y. *Biochimie* 53, 1111–1114 (1971).
- 14 Kourilsky, P., Mercereau, O., Gros, D. & Tremblay, G. Y. *Biochimie* 56, 1215–1221 (1974).
- 15 Kourilsky, P., Bourguignon, M. F. & Gros, F. in *The Bacteriophage Lambda* (ed. Hershey, A. D.) 647–666 (Cold Spring Harbor, New York, 1971).
- 16 Herskowitz, I. A. *Rev. Genet.* 7, 289–324 (1973).
- 17 Mercereau-Pujalon, O. & Kourilsky, P. *J. molec. Biol.* 108, 733–751 (1976).
- 18 Cameron, J. R., Panasenko, S. M., Lehman, I. R. & Davis, R. W. *Proc. natn. Acad. Sci. U.S.A.* 72, 3416–3420 (1975).

Molecular characterisation of the tyrosine tRNA genes of yeast

MANY of the tRNA genes of yeast are observable genetically as nonsense suppressors¹. In particular, the set of unlinked suppressor loci *SUP2* to *SUP8* and *SUP11* have been shown to insert tyrosine at nonsense codons in yeast iso-1-cytochrome c^{2,3}. Direct biochemical evidence for the presence of suppressor tRNA in yeast strains bearing these suppressor genes has been obtained using *in vitro* translation systems^{4,5} and by the demonstration that a new minor tyrosine tRNA species with a suppressor anti-codon appears in a *SUP5* strain⁶. Although these

findings suggest that there are eight different loci in yeast which code for tRNA^{tyr}, only a single nucleotide sequence has been observed for tyrosine tRNA in wild-type yeast strains⁷. Thus it seems that all eight genes may share a common base sequence, at least in their structural regions. Because the yeast tRNA^{tyr} *SUP* genes occupy known positions on the genetic map⁸ and are complementary to a well characterised RNA molecule they are attractive candidates for molecular analysis. As a first step, we have labelled yeast tRNA^{tyr} with ¹²⁵I and have hybridised this probe to electrophoretically fractionated *Eco*RI fragments of yeast DNA; eight *Eco*RI fragments of different sizes hybridise to tRNA^{tyr}.

Figure 1 shows the molecular weight distribution of *Eco*RI-cleaved yeast DNA and documents the efficiency with which the DNA can be transferred from the gel to a nitrocellulose filter. In addition to the three prominent bands of molecular weight (mw) between 1 and 2 × 10⁶, which contain ribosomal cistrons, there is a continuous distribution of DNA sizes up to > 10⁷ mw (ref. 9). Comparison of the two curves in Fig. 1b shows that *Eco*RI fragments between 5 × 10⁵ and 5 × 10⁶ mw are transferred to nitrocellulose¹⁰ with 80% efficiency and that the transfer efficiency decreases significantly for DNA fragments larger than 5 × 10⁶ mw. The structure of the DNA banding pattern is retained remarkably well through the transfer process, as may be seen by comparing the gel stain pattern and filter autoradiogram (Fig. 1a).

DNA from yeast strain B596 (ref. 11) was *Eco*RI-restricted, electrophoresed on a 0.7% Agarose gel, and then transferred, as denatured DNA, to nitrocellulose filter strips¹⁰. After drying, the filter strips were hybridised to ¹²⁵I-labelled tRNA^{tyr}. The tRNA^{tyr} used for hybridisation was partially purified from total yeast tRNA by the acylation-BD cellulose procedure of Gillam *et al.*¹². The derivatised, ³H-tyrosine-aminoacylated tRNA^{tyr}

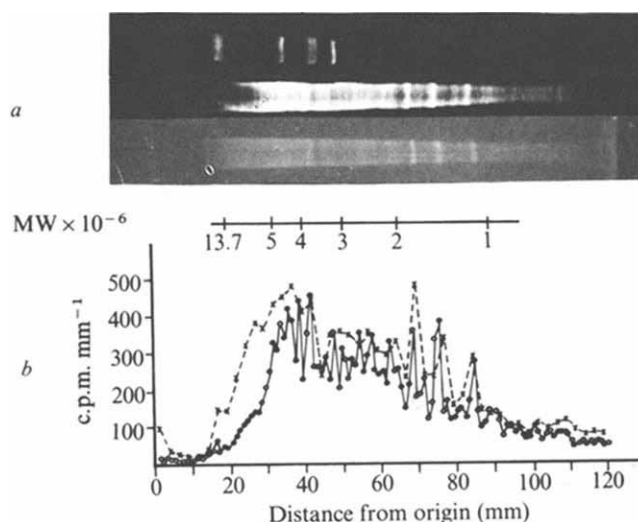


Fig. 1 Agarose gel fractionation of *Eco*RI-cleaved total yeast DNA. *a*, High molecular weight ¹⁴C-labelled yeast DNA was prepared by lysing glucosylase-treated cells in sarkosyl and purifying the released DNA by velocity sedimentation through sucrose and isopycnic banding in CsCl. A limit *Eco*RI digest of this yeast was electrophoresed on a 0.7% Agarose slab gel, stained with ethidium bromide, and then transferred to nitrocellulose by the procedure of Southern^{10,11}. The top strip shows the banding pattern of *Eco*RI-cleaved DNA from bacteriophage λ , electrophoresed on the same gel. The middle strip shows an autoradiogram of the nitrocellulose strip containing the yeast *Eco*RI fragments. The bottom strip is a photograph of the yeast ethidium bromide staining pattern. *b*, On a parallel well of the same gel described in (*a*) an identical aliquot of *Eco*RI-cleaved yeast DNA was electrophoresed. The gel was sliced and the individual slices counted (broken line). The nitrocellulose strip whose autoradiogram is shown in (*a*) was also sliced and counted (solid line). The counting efficiency was the same for the gel and nitrocellulose filter slices so the two curves in (*b*) are a direct measure of the efficiency with which the DNA was transferred to the filter.

was eluted from BD cellulose in 1M NaCl, 11% ethanol. It was then iodinated with ^{125}I in the presence of peroxidase and H_2O_2 . In our hands this procedure gave material with a specific activity two orders of magnitude higher than we could achieve by *in vivo* labelling with ^{32}P . An autoradiogram of a hybridised filter strip is shown in Fig. 2a; eight discrete hybridisation bands are present.

To test conclusively whether tRNA^{tyr} molecules were responsible for the eight hybridisation bands observed in Fig. 2a, competition hybridisation was performed using cold competitor tRNA^{tyr} that had been highly purified by a procedure differing from that used to prepare tRNA^{tyr} for iodination. Specifically, the competitor was purified by sequential chromatography on BD cellulose, first as uncharged tRNA^{tyr} then as aminoacylated tyr-tRNA^{tyr} (ref. 13); finally, the material was further purified by chromatography on Sepharose¹⁴ using a reverse ammonium sulphate gradient. Competition of the ^{125}I -tRNA hybridised to all eight bands is observable at an unlabelled tRNA^{tyr} concentration of $0.1 \mu\text{g ml}^{-1}$; competition is virtually complete at $1.0 \mu\text{g ml}^{-1}$. The estimated concentration of ^{125}I -labelled tRNA^{tyr} in the hybridisations was $0.1 \mu\text{g ml}^{-1}$. In subsequent experiments the same eight band pattern shown in Fig. 2a was obtained by hybridisation of yeast tRNA^{tyr} which had been purified to apparent homogeneity before labelling with ^{125}I . These data strongly suggest that each of the eight observed bands arises from hybridisation to a tRNA^{tyr} gene.

The molecular weights of the eight *Eco* RI fragments that hybridise to ^{125}I -labelled tRNA^{tyr} were estimated from their electrophoretic mobilities. Band positions on autoradiograms

Fig. 2 Hybridisation of ^{125}I -labelled tRNA^{tyr} to yeast *Eco* RI fragments. Iodinated tRNA^{tyr} with a specific activity of $>5 \times 10^7$ c.p.m. μg^{-1} was hybridised to DNA immobilised on nitrocellulose strips in 60% formamide, 1.15 M sodium acetate, pH 6 at 40°C for 3 h. The hybridisation conditions were similar to those used by Birnstiel¹⁶ for tRNA hybridisations. After extensive rinsing and RNase treatment, autoradiograms of the filter strips were recorded. Hybridisation was carried out in the presence of a, 0; b, $0.1 \mu\text{g ml}^{-1}$; and c, $1 \mu\text{g ml}^{-1}$ of purified, unlabelled tRNA^{tyr}.

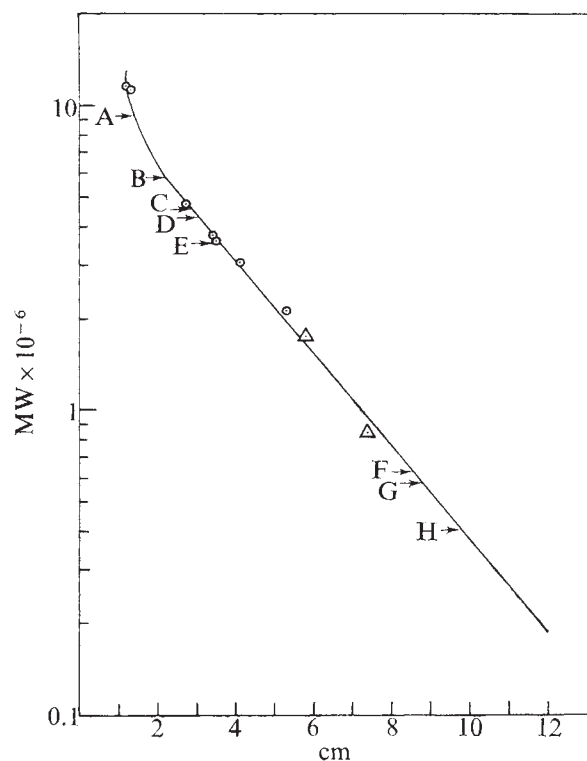
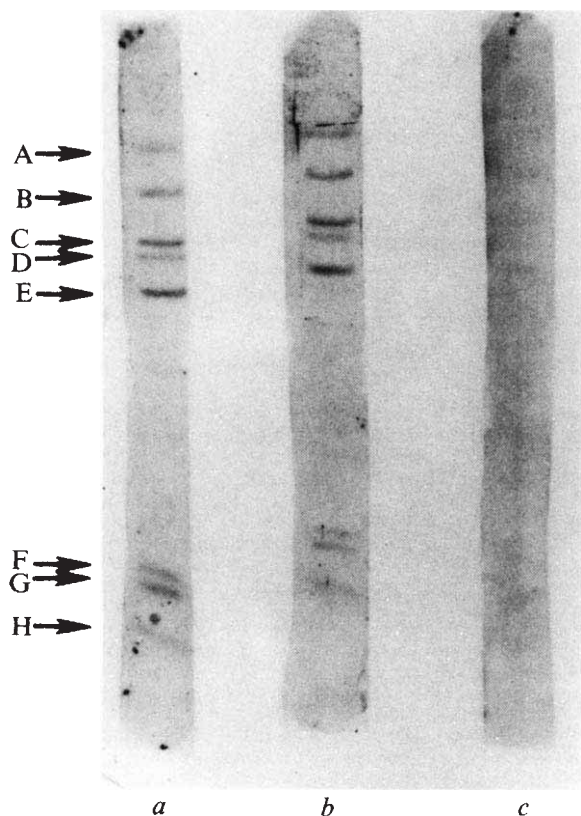


Fig. 3 Agarose gel mobilities of the yeast *Eco* RI fragments that hybridise to tRNA^{tyr}. The arrows show the positions of the eight hybridising fragments, A-H. λ *Eco* RI fragments, $\circ$; yeast rDNA fragments I and III, Δ . Molecular weights of the λ fragments are from ref. 15; values for the yeast rDNA fragments are calculated from data in ref. 16.

of the nitrocellulose filters were standardised with ^{14}C -labelled *Eco* RI fragments of bacteriophage λ (ref. 15) and also with yeast rDNA fragments I and III, which were located by hybridisation with ^{32}P -labelled 18 and 28S rRNA¹⁶. A plot from a typical gel is shown in Fig. 3. Approximate molecular weights for the hybridising fragments ($\times 10^3$) are as follows: A ≈ 9 ; B, 5.8; C, 4.5; D, 4.2; E, 3.5; F ≈ 0.6 ; G ≈ 0.6 ; H ≈ 0.4 .

Because the base sequence of yeast tRNA^{tyr} does not contain an *Eco* RI site, each DNA sequence that hybridises to this tRNA should lie within a single *Eco* RI fragment. Consequently, the number of hybridisation bands observed should equal the number of tRNA^{tyr} genes, which seems to be eight. The length of the *Eco* RI fragment which contains each of these eight genes will be determined by the nature of the chromosomal DNA sequences which lie to either side of the tRNA^{tyr} gene. Our results indicate that this neighbouring DNA has different characteristics for different tRNA genes, since the length of accompanying DNA varies from less than 1,000 to more than 12,000 base pairs for different restriction fragments which hybridise ^{125}I -tRNA^{tyr}.

An indication that one of the eight loci differs from the others within the tRNA gene is given by our hybridisation results (Fig. 2a and b). Band D contains noticeably less hybridised tyrosine tRNA than the more intense bands C and E on either side. We have observed band D to be consistently less intense than its immediate neighbours in a large number of experiments using DNA from several different haploid and diploid yeast strains. The data in Fig. 1b indicate that systematic loss of band intensity at low and high molecular weights is expected due to incomplete transfer of DNA to the nitrocellulose. This effect fails to explain the diminished intensity of band D, however, which is in a region of high, constant efficiency and is closely bracketed by more intense bands. Although differences in the number of tRNA^{tyr} genes at each locus remain a possible source of variable band intensity, the existing genetic data favour the hypothesis that each locus contains a

single copy¹⁷. To identify which suppressor gene corresponds to each of these bands, the procedures described here can be applied to DNA from yeast strains either deleted for particular suppressor loci or aneuploid for one of the relevant chromosomes. Further progress in the structural analysis of individual tRNA¹⁷ genes and the adjoining DNA sequences will depend on the successful molecular cloning of the *Eco* RI fragments that we have identified. The ¹²⁵I-tRNA probe described here should be well-suited to screening plaques or colonies for the presence of tDNA¹⁷ sequences in recombinant genomes.

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- ¹ Hawthorne, D. C. & Leupold, U. *Curr. Top. Microbiol. Immun.* **64**, 1–47 (1974).
- ² Gilmore, R. A., Stewart, J. W. & Sherman, F. *J. molec. Biol.* **61**, 157–173 (1971).
- ³ Sherman, F., Liebman, S. W., Stewart, J. W. & Jackson, M. *J. molec. Biol.* **78**, 157–168 (1973).
- ⁴ Capecchi, M. R., Hughes, S. H. & Wahl, G. M. *Cell* **6**, 269–277 (1975).
- ⁵ Gesteland, R. F. *Cell* **7**, 381–390 (1976).
- ⁶ Piper, P. W. *et al. Nature* **262**, 757–761 (1976).
- ⁷ Madison, J. T. & Kung, H. K. *J. biol. Chem.* **242**, 1324–1330 (1967).
- ⁸ Mortimer, R. K. & Hawthorne, D. C. *Genetics* **74**, 33–54 (1973).
- ⁹ Nath, K. & Bollon, A. P. *Nature* **257**, 155–157 (1975).
- ¹⁰ Southern, E. M. *J. molec. Biol.* **98**, 503–517 (1975).
- ¹¹ Sherman, F., Stewart, J. W., Jackson, M., Gilmore, R. A. & Parker, J. H. *Genetics* **77**, 255 (1974).
- ¹² Gillam, I., Blew, D., Warrington, R. C., von Tigerstrom, M. & Tener, G. M. *Biochemistry* **7**, 3459–3468 (1968).
- ¹³ Maxwell, I. H., Wimmer, E. & Tener, G. M. *Biochemistry* **7**, 2629–2634 (1968).
- ¹⁴ Holmes, W. M., Hurd, R. E., Reid, B. R., Rimerman, R. A. & Hatfield, G. W. *Proc. natn. Acad. Sci. U.S.A.* **72**, 1068–1071 (1975).
- ¹⁵ Helling, R. B., Goodman, H. M. & Boyer, H. W. *J. Virol.* **14**, 1235–1244 (1974).
- ¹⁶ Kramer, R. A., Cameron, J. R. & Davis, R. W. *Cell* **8**, 227–232 (1976).
- ¹⁷ Mortimer, R. K. *Genetics Suppl.* **G1-1**, 329–334 (1969).
- ¹⁸ Birnstiel, M. L., Sells, B. H. & Pardoll, I. F. *J. molec. Biol.* **63**, 21–39 (1972).

Nucleotide sequence of the yeast 5S ribosomal RNA gene and adjacent putative control regions

IN bacteria, the sequence of several promoters has been determined and some of the features of their interaction with RNA polymerase, repressors and other regulatory molecules are beginning to be determined^{1,2}. In eukaryotes, however, the isolation and analysis of the DNA structures involved in the regulation of transcription has not yet been achieved. Recent advances in techniques for cloning eukaryotic DNA in bacterial plasmids³ have allowed the isolation of genetic segments whose structure and function can be studied in detail. Using these methods we have isolated a DNA fragment containing the 5S ribosomal RNA gene. The 5S RNA seems to be a primary transcript in yeast^{4–6}; therefore the flanking regions of the 5S gene should contain the putative promoter and termination regions. We report here the nucleotide sequence of *Saccharomyces cerevisiae* 5S DNA and these flanking regions.

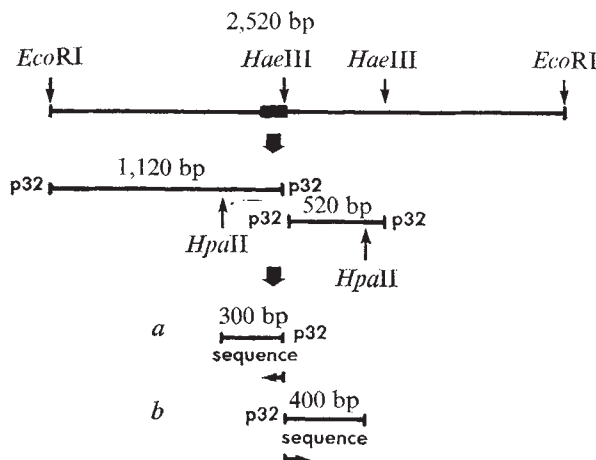
Yeast DNA and the plasmid vector pSF2124 (ref. 7) were digested with *Eco*RI, ligated, and used to transform a derivative of *E. coli* K12, DG-75 (a F[–] derivative of DG73 ref. 8)). Recombinant clones were selected by ampicillin resistance and inability to produce colicin E1. Recombinants containing 5S rDNA were selected by hybridisation with purified yeast 5S rRNA. A clone (pDB25) containing

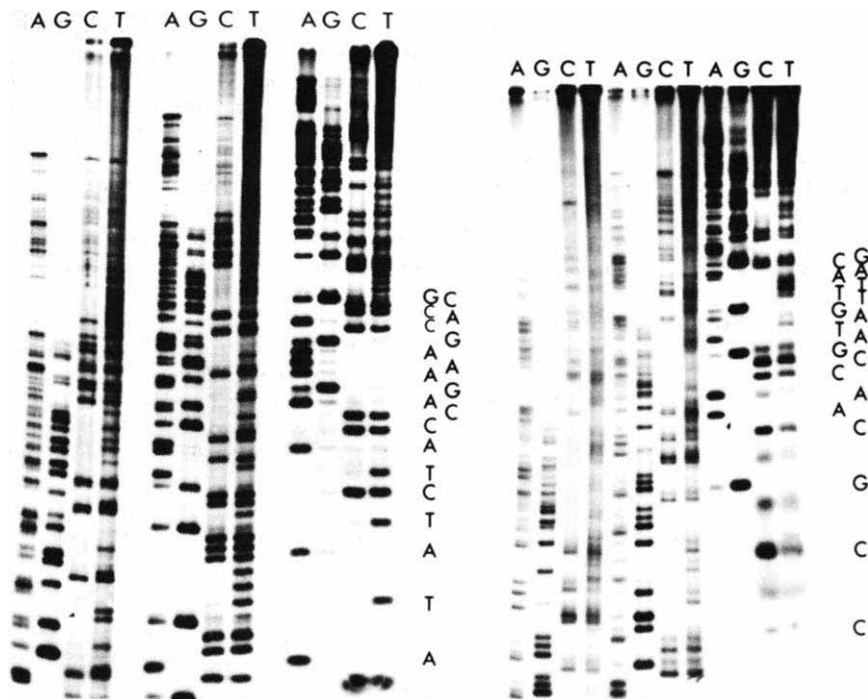
a 2,520 base pair yeast DNA fragment which hybridises with 5S rRNA was obtained.

The tentative position of the 5S rRNA gene within the 2,520 base pair fragment was determined by restriction mapping with endonucleases *Hae*III, *Alu*I and *Hpa*II and by hybridisation of the restriction fragments with ¹²⁵I-labelled yeast 5S rRNA (P.V. *et al.* in preparation). The sequence of 5S RNA of *S. cerevisiae*⁴ and of the related species, *S. carlsbergensis*⁵, predicts a *Hae*III site eight base pairs from the 3' end of the gene. Therefore as indicated in Fig. 1, we isolated the two *Hae*III fragments (1,120 and 520 base pairs, respectively) which encompass the 5S rRNA gene, labelled them at their 5' ends with γ -³²P-ATP and polynucleotide kinase, and digested them with *Hpa*II. Two fragments, each containing a complementary portion of the 5S RNA gene, of 300 (a) and 400 (b) base pairs were isolated by preparative gel electrophoresis and partially sequenced from their ³²P-labelled *Hae*III ends. DNA was sequenced by using the chemical cleavage technique developed by Maxam and Gilbert⁹. Figure 2 shows an autoradiogram of the electrophoretic separation of oligonucleotides resulting from base specific cleavages of fragments a and b. The sequence can be directly read from the autoradiogram as exemplified in the figure for the first 10 nucleotides. The experiment with fragment a (Fig. 2a) generated a sequence comprising 112 bases from the 5S rRNA gene and 61 bases from the region adjacent to the 5' end (termination region). An *Alu*I fragment of 160 base pairs containing this region was also sequenced (P.V. unpublished). The sequence of fragment b (Fig. 2b) furnished a sequence of 68 base pairs, 8 from the 5S gene and 60 from the region adjacent to its 3' end (initiation region). The complete sequence of the 5S RNA gene and adjacent regions is shown in Fig. 3. The sequence of other regions, comprising about 70%, of the 2,520 base pair fragment has been completed and will be reported elsewhere.

The sequence of most of the 5S RNA from *S. cerevisiae* has been reported by Miyazaki⁴. However, a small region around the 35th position (from the 5' end) remained uncertain⁴. The sequence we have determined for the 5S RNA

Fig. 1 Strategy for isolation and sequencing of yeast DNA fragments containing the 5S rRNA gene. The 2,520 base pair yeast DNA insert containing the 5S RNA gene was excised from the hybrid plasmid, pDB25, by digestion with *Eco*RI and preparatively separated from the bacterial vector by sucrose gradient centrifugation (5–20% sucrose, 20 h, SW-27 rotor, 20 °C). The *Hae*III fragments were prepared by digestion of 100 μ g of the yeast DNA insert with *Hae*III and separated by preparative slab gel electrophoresis (5% acrylamide, 50 mM Tris-borate (pH 8.3), 1 mM EDTA, at constant 10 V cm^{–1}). After elution from the gel, the isolated fragments were labelled at their 5' ends (T4 polynucleotide kinase and ³²P-ATP (ref. 9)) and digested with *Hpa*II. Fragments a and b were separated by preparative gel electrophoresis (as above) and sequenced from their labelled 5' ends.





for adenine, guanine, cytosine, and thymine, respectively. The reading of the first 10 bases is illustrated at the right of each autoradiogram. The first two nucleotides of the labelled 5' end of fragment *b* ran off the gel and are determined from the specificity of *Hae*III. Fragment *a* is at the left, fragment *b* is at the right.

gene confirms Miyazaki's results and establishes the sequence of this region. Comparison with the sequence of 5S RNA from *S. carlsbergensis*⁵ indicates small differences. Some may be due to species differences while others are probably due to minor errors in the proposed RNA sequence. Four of these changes occur in a region where, according to Hindley and Page⁵, the sequence was uncertain.

The nucleotide sequence of the 60 base pairs adjacent to the 3' end of the 5S gene should contain the site where RNA polymerase attaches to DNA to initiate transcription and possibly the sites for other regulatory molecules. This region may be compared with the known promoter regions of prokaryotes. Pribnow¹⁰ has observed that several pro-

karyotic promoters contain an AT-rich heptanucleotide centred 9 to 10 bases before the transcription initiation site. Mutations that increase promotion in the *lac* operon are of the type GC to AT and occur in these regions or the immediate vicinity¹. The putative promoter in yeast contains an AT-rich sequence, GTTAAAC, centred 8 bases from the start of 5S RNA transcription and bears some similarity to the 'Pribnow box'. The yeast promoter does not possess the general sequence homology (TTGA and GTTG) found in prokaryotes in a region centred about 35 bases from the start of the gene, nor palindromes which in some prokaryotic promoters have been identified as binding sites for regulatory proteins^{1,2}. Fedoroff and Brown (personal communication) have recently determined the structure of the

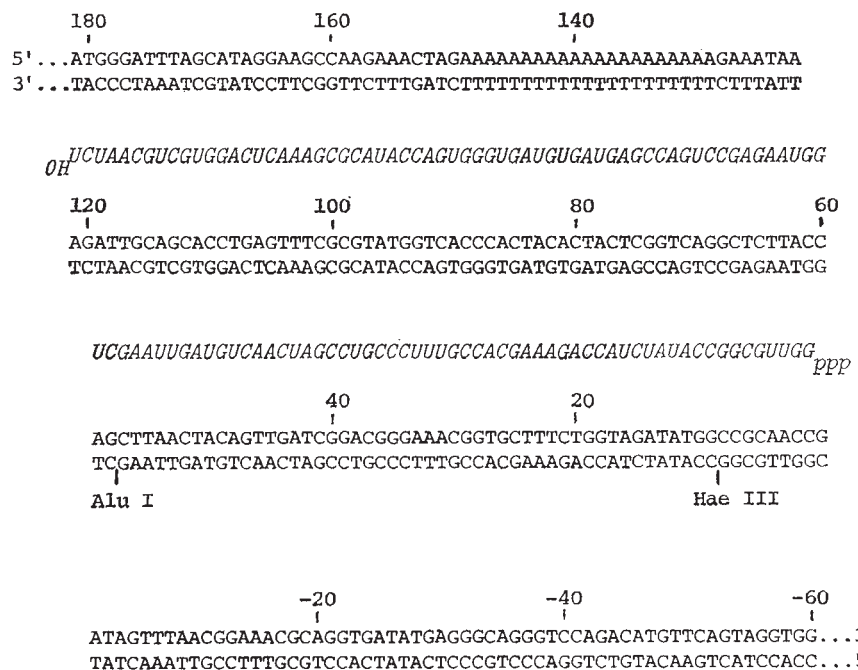


Fig. 3 Nucleotide sequence of *S. cerevisiae* 5S rRNA gene and adjacent regions. The corresponding 5S rRNA sequence is shown in italics.

promoter region for *Xenopus* oocyte 5S RNA genes. There is no obvious relationship between the structures of this region and the region we have sequenced. The fundamental structural relationships which determine transcription promotion in eukaryotes are as yet difficult to perceive.

In contrast, at the end of the 5S gene there is a striking stretch of As in the coding strand (Fig. 3). We believe this to be a termination signal for RNA polymerase. Termination in other systems may be related to A-rich regions. For example, variable runs of Us have been found at the 3' end of 4S (ref. 11) and 6S (ref. 12) RNAs of λ phage, a small RNA from phage ϕ 80 (ref. 13) and the attenuator region of tryptophan mRNA of *E. coli*¹⁴. There is no evidence for transcription of this A region in yeast; however, transcription and rapid processing has not been eliminated. In eukaryotes, Brown and Brown¹⁵ have found a much shorter AT-rich region at the 5' end of the *Xenopus* 5S RNA gene and postulated the sequence $\begin{matrix} \text{TTTT} \\ \text{AAAA} \end{matrix}$ as part of a transcription termination signal.

RNA polymerase binding and *in vitro* transcription experiments will be necessary to determine the role of these sequences in selective gene transcription.

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- ¹ Gilbert, W. in *RNA Polymerase* (eds Losick, R. & Chamberlin, M.) 193–205 (Cold Spring Harbor Press, 1976).
- ² Dickson, R. C., Abelson, J., Barnes, W. M. & Reznikoff, W. S. *Science* **187**, 27–35 (1975).
- ³ Morrow, J. F., Cohen, S. N., Chang, A. C. Y., Boyer, H. W., Goodman, H. M. & Helling, R. B. *Proc. natn. Acad. Sci. U.S.A.* **71**, 1743–1747 (1974).
- ⁴ Miyazaki, M. J. *Biochem.* **75**, 1407–1410 (1974).
- ⁵ Hindley, J. & Page, S. M. *FEBS Lett.* **26**, 157–160 (1972).
- ⁶ Trapman, J. & Planta, R. J. *Biochim. biophys. Acta* **414**, 115–125 (1975).
- ⁷ So, M., Gill, R. & Falkow, S. *Molec. gen. Genet.* **142**, 239–249 (1975).
- ⁸ Polisky, B., Bishop, R. J. & Gelfand, D. H. *Proc. natn. Acad. Sci. U.S.A.* **73**, 3900–3904 (1976).
- ⁹ Maxam, A. & Gilbert, W. *Proc. natn. Acad. Sci. U.S.A.* **74**, 560–564 (1977).
- ¹⁰ Pribnow, D. *Proc. natn. Acad. Sci. U.S.A.* **72**, 784–788 (1975).
- ¹¹ Sklar, J., Yot, P. & Weissman, S. *Proc. natn. Acad. Sci. U.S.A.* **72**, 1817–1821 (1975).
- ¹² Dahlberg, J. & Blattner, F. *Fed. Proc. (Abstr.)* **32**, 664 (1973).
- ¹³ Piecznik, G., Barrell, B. G. & Gelfand, M. L. *Archs Biochem. Biophys.* **152**, 152–165 (1972).
- ¹⁴ Bertrand, K. *et al. Science* **189**, 22–26 (1975).
- ¹⁵ Brown, R. D. & Brown, D. D. *J. molec. Biol.* **102**, 1–14 (1976).

Promotor region for yeast 5S ribosomal RNA

WE have sequenced the DNA region immediately preceding the structural gene for the 5S ribosomal RNA of yeast (*Saccharomyces cerevisiae*). Since the 5S RNA, a component of the larger ribosomal subunit, has a 5' triphosphate^{1,2}, this molecule is likely to be the direct product of the gene, unmodified by maturation at the 5' end. Thus the DNA sequence immediately before this region must contain the recognition sequences where the RNA polymerase binds to begin synthesis, that is, this sequence must represent a eukaryotic promoter.

T. D. Petes, P. Wensink and D. Botstein (unpublished) isolated a series of plasmids containing sheared DNA from a diploid yeast strain (A364a+D4, provided by L. H. Hartwell). Digestion of those containing yeast rDNA with *EcoRI* generated a map of the ribosomal area (T. D. Petes, L. Hereford and K.G.S., in preparation), shown in Fig. 1. The map was derived by noting the associations

between RI fragments in different clones carrying some but not all of the ribosomal repeat unit, and hybridisation of mature 5S, 5.8S, 18S and 28S rRNAs to the individual *EcoRI* fragments. Only fragment B, the second largest *EcoRI* fragment, 2,600 base-pairs long, hybridised to the 5S RNA. Sequential cleavage with *SmaI* (from *Serratia marcescens*) and *EcoRI* then oriented RI fragment B with respect to its neighbours.

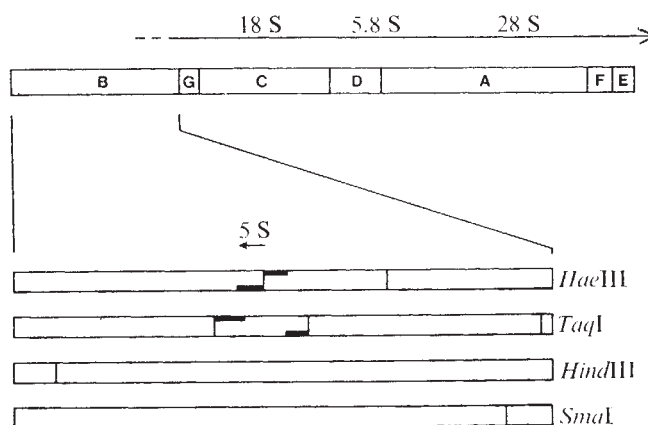
The nucleotide sequence of yeast 5S rRNA¹ suggested several restriction endonuclease sites in the structural gene, the one of primary interest being a *HaeIII* (from *Haemophilus aegyptius*) cut eight nucleotides from the 5' end of the RNA. To construct a restriction map of the *EcoRI* B fragment, we end-labelled it with polynucleotide kinase and λ -³²P-ATP, cut the fragment close to either one or the other end with *SmaI* or *HindIII* (from *Haemophilus influenzae*) (Fig. 1), and then examined partial digests of the resulting long, uniquely terminally labelled fragments with a series of restriction enzymes³. Sites for two which cleaved fragment B, *HaeIII* and *TaqI* (from *Thermus aquaticus*), are shown in Fig. 1.

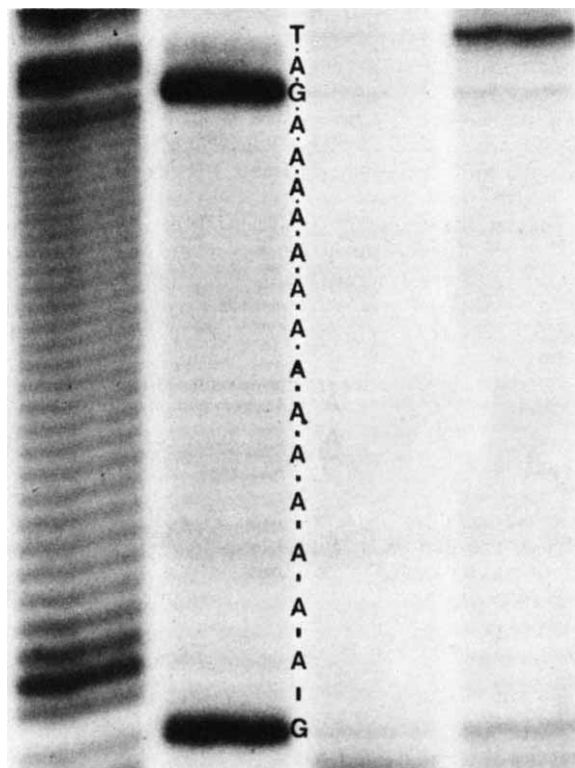
We then sequenced⁴ the ends of all of the *HaeIII* fragments in order to identify which fragment junction lay within the 5S rRNA gene. From this *HaeIII* junction, we then sequenced out 100 bases before the gene, and on the other side, the gene itself (Fig. 1). Sequences at the extremities of this region were confirmed by sequencing towards the gene on the complementary strands from each of the two outlying *TaqI* cleavage sites. Figure 2 is an autoradiograph from one of these strands: a striking poly(dA) run that terminates the gene. Figure 3 shows the DNA sequence derived¹ from analogous patterns on the four strands, and the corresponding 5S rRNA.

Our structure for 5S rDNA confirms most of the RNA sequences worked out by Miyazaki¹ for *S. cerevisiae* and by Hindley and Page² for *S. carlsbergensis*, and generally agrees with one from *Torulopsis utilis*⁵. Except for two base changes (at positions 61 and 73) in *T. utilis*, the differences between the sequences are most likely to be minor errors in RNA sequencing: failures in ordering a series of pyrimidines within a single T₁ fragment (an extensive comparison of these sequences is in ref. 6).

The restriction maps and nucleotide sequences determine the direction of transcription of the 5S gene to be from right to left on the *EcoRI* B fragment, as shown, and thus

Fig. 1 Restriction endonuclease cleavage map of cloned *S. cerevisiae* rDNA. The order of fragments (A–G) released from the ribosomal repeat unit by *EcoRI* is shown at the top, and at the bottom, sites in fragment B for four other endonucleases. Arrows represent precursor RNAs, and thickened lines in the lower map indicate end-labelled DNA strands which were sequenced. (*EcoRI* map courtesy of T. D. Petes, L. Hereford, and K. G. Skryabin, in preparation).





-100 -80 -60 -40 -20
 AAA TATAATAAAAATTGTCCTCCACCCATAACACCTCTCACTCCCACCTACTGACCATGTCTGGACCCCTGCCCTCATATCACCTGCGTTTCCG
 TTTATATTATTTTAAACAGGAGGTGGTATTGTGGAGAGTGAGGGTGGATGACTTGACAGACCTGGGACGGGAGTATAGTGGACGCAAAGGC

pppGGUUGCGGCCAUAUCUACCAGAAAGCACCGUUUCCCGUCCGAUCAAUGUAGUUAAUGUGGUAAGAGCCUGACCGAUAAGUGUAGUGG

TTA AACTATCGGTTGCGGCCATATCTACCAGAAAGCACCGTTTCCCGTCCGATCAACTGTAGTTAAGCTGGTAAGAGCCTGACCGAGTAGTGTAGTGG
 AATTTGATAGCCAACGCCGGTATAGATGGTCTTTCGTGGCAAAGGGCAGGCTAGTTGACATCAATTCGACCATTCTCGGACTGGCTCATCACC

HaeIII

GUGACCAUACGCCAAACUCAGGUGCUGCAAUUCU_{OH}

100 120 140 160 180
 GTGACCATAACGCGAAACTCAGGTGCTGCAATCTTTATTTCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCTAGTTTCTTGGCTTCCTATGCT
 CACTGGTATGCGCTTTGAGTCCACGACGTTAGAAATAAAGAAAAAAAAAAAAAAAAAAAAAAAAAAGATCAAAGAACCGAAGGATACGA

The yeast sequence does not resemble any of the common

Fig. 3 Nucleotide sequences before, within, and beyond the *S. cerevisiae* gene for 5S rRNA.

pppG

CCTCCACCCATAACACCTCTCACTCCACCTACTGAACATGTCTGGACCCTGCCCTCATATCACCTGCGTTTCCGTTAACTATCG
GGAGGTGGGTATTGTGGAGAGTGAGGGTGGATGACTTGTACAGACCTGGGACGGGAGTATAGTGGACGCAAGGCAATTTGATAGC

CCT C C CATA CACCT
GGA G G GTAT GTGGA

CCT C C CATA CACCT
GGA G G GTAT GTGGA

T A ATAA A T T A T A TA T AA AT T T A T T ATAT A T TTT TTAAA TAT
A T TATT T A A T A T AT A TT TA A A T A A TATA T A AAA AATTT ATA

A A AA A A A A GAA A G GGA G A A A G G G AAA A G
GGAGG GGG A G GGAGAG GAGGG GGA GA G A AGA GGGA GGGAG A AG GGA G AAAGG AA GA AG

Fig. 4 Nucleotide distribution in the promoter region for *S. cerevisiae* 5S rRNA. The guanosine triphosphate which initiates the RNA chain is shown above the DNA sequence at the extreme right. Underneath are shown a 14-nucleotide-pair repeating sequence and the distributions of A-T pairs and purine nucleotides in this region.

elements in the prokaryotic promoters; further exploration will be necessary to identify the critical nucleotides and regions with which the yeast polymerase interacts.

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- ¹ Miyazaki, M. *J. Biochem. (Tokyo)* **75**, 1407-1410 (1974).
- ² Hindley, J. & Page, S. M. *FEBS Lett.* **26**, 157-160 (1972).
- ³ Smith, H. O. & Birnstiel, M. L. *Nucleic Acids Res.* **3**, 2387-2398 (1976).
- ⁴ Maxam, A. M. & Gilbert, W. *Proc. natn. Acad. Sci. U.S.A.* **74**, 560-564 (1977).
- ⁵ Nishikawa, K. & Takemura, S. *FEBS Lett.* **40**, 106-109 (1974).
- ⁶ Gilbert, W., Maxam, A. M., Tizard, R. & Skryabin, K. G., in *Eukaryotic Gene Expression* (eds Abelson, J. & Wilcox, G. W.) (Academic, New York, 1977, in the press).
- ⁷ Weinmann, R. & Roeder, R. G. *Proc. natn. Acad. Sci. U.S.A.* **71**, 1790-1794 (1974).
- ⁸ Gilbert, W. in *RNA Polymerase* (eds Losick, R. & Chamberlin, M.) 193-205 (Cold Spring Harbor Laboratory, 1976).
- ⁹ Ptashne, M. *et al. Science* **195**, 156-161 (1976).
- ¹⁰ Valenzuela, P., Bell, G. I., Masiarz, F. R., DeGennaro, L. J. & Rutter, W. J. *Nature* **267**, 641-643 (1977).

Kinetics of rhodopsin at room temperature measured by picosecond spectroscopy

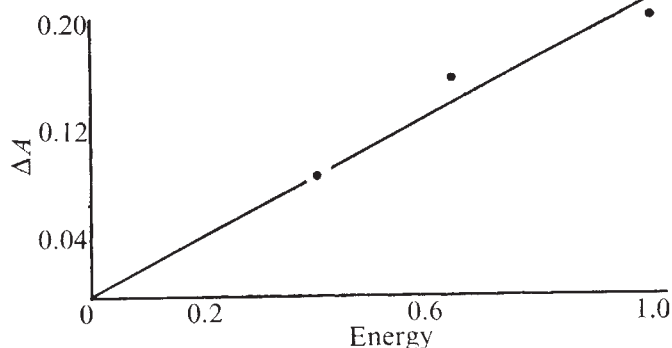
The photoinduced intermediates of the visual photoreceptor rhodopsin have been studied by optical spectroscopy in low temperature glasses and at room temperature. The primary changes in the absorption spectrum of rhodopsin were first observed at 77 K by Yoshizawa and Kito¹ then by Grellman *et al.*² and Yoshizawa and Wald³. The first intermediate observed, prelumirhodopsin (bathorhodopsin) exhibits a maximum at 545 nm and has been assigned to the primary isomerisation step of the rhodopsin polyene chromophore. The room temperature kinetics of formation and decay of an intermediate species absorbing at 560 nm were measured by Busch *et al.*⁴ using picosecond spectroscopy. This original work revealed that this species, assigned as prelumirhodopsin, was formed within 6×10^{-12} s and decayed with a lifetime of 3×10^{-8} s. Several technical innovations in picosecond spectroscopy⁵⁻⁷ now enable us to monitor spectra changes between 400 nm and 1,500 nm as a function of time with ± 0.02 absorbance unit resolution. We report here a complete difference spectrum for prelumirhodopsin

at room temperature mapped on a picosecond time scale. These studies show that the loss of rhodopsin and the rise of prelumirhodopsin are kinetically consistent. If care is taken to avoid photon saturation no 'ghost' species appear in the region of 400-440 nm as has been reported previously^{8,9}.

Bovine rhodopsin samples, solubilised in Ammonyx LO detergent, were prepared as described in ref. 10. The optically clear samples contained between 1 and 2×10^{-4} M rhodopsin (that is, 4-7 absorbance units per cm at 500 nm) and the absorbance ratios for 400 nm/500 nm were 0.20 ± 0.02 . For the experiments described, special care was taken to shield the sample from all spurious light emitted by the flashlamp or amplifier system, and all the experiments were conducted in the dark or under dim red light. Measurements were made in a 2-mm optical path length cell. We estimate that less than 15% of the sample in the optical path was bleached per excitation pulse. For each kinetic observation at a given wavelength, the sample was mixed between successive shots and completely replaced after five shots.

The experimental apparatus is described in detail in ref. 5. A single pulse is selected from the train of pulses generated by a Nd³⁺ glass laser emitting at 1,060 nm. The pulse is amplified and the frequency doubled to 530 nm. Thereafter it is split into two parts; one acting as the excitation pulse and the other inducing the interrogation continuum in a benzene or alcohol cell. This continuum is segmented into several spatially equidistant pulses by an echelon thereby providing the means for observing time resolved absorption spectra. These continuum pulses, which are too weak in intensity to cause any measurable excitation, are detected by a SIT vidicon and subsequently analysed, averaged, and displayed by a Nova computer. Care is taken to avoid dispersion and saturation effects by following the practice of timing the excitation pulse with

Fig. 1 Absorbance changes plotted against pulse energy. A straight line with an intercept at zero would indicate that no saturation occurred within this region of excitation pulse energy where our experiments were performed.



each wavelength and of making certain that the vidicon was used in its linear range.

Particular care was taken to eliminate photon saturation of the sample since rhodopsin, as most other highly sensitive biological molecules, can easily be saturated by an intense light pulse which may produce erroneous multiphoton events. Changes in ΔA were examined as a function of the laser pulse intensity as shown in Fig. 1 and data were used only when they fell within the linear range of this plot. An additional indication that the system is not saturating is shown in Fig. 2 in which the photoinduced ΔA increases proportionally with increasing sample concentration at constant excitation intensity.

Excitation of rhodopsin by a single, 6-ps 530-nm pulse resulted in the bleaching of the rhodopsin band and simultaneous formation of prelumirhodopsin. The entire difference spectrum, recorded 60 ps after excitation is plotted in Fig. 2. This spectrum is characterised by:

- (1) The bleached rhodopsin band at 430–510 nm with a maximum ΔA at 485 nm.
- (2) Formation of a band at 530–680 nm with a maximum ΔA at 580 nm.
- (3) An approximate isosbestic point at 525 nm.

This difference spectrum is in approximate agreement with the previously reported data for low temperature glasses in digitonin², rhodopsin in Ammonyx LO (ref. 10), and rhodopsin in intact retina¹¹. The kinetics indicate that the 485-nm band bleaches and the 580-band maximum arises within 6 ps as shown in Fig. 3.

There is one striking difference between the room temperature picosecond spectrum and those observed in low temperature glasses. The ratio of maximum and minimum ΔA in the difference spectrum observed at room temperature is 1 : 1. At low temperatures under a photo-stationary equilibrium, which is carefully established to exclude formation of isorhodopsin, this ratio is 2.5 : 1 or 3 : 1 (refs 10, 11). In addition the isosbestic point is located at 525 nm at room temperature versus 515 nm in low temperature glasses.

The source of this discrepancy is not clear. It is possible that temperature affects the band width and shape to some extent, however, it is more probable that at room temperature the excited rhodopsin decays not only to a prelumirhodopsin state but back to rhodopsin or other states via channels which are not open at low temperatures due to the rigidity of the species. To elucidate the operative mechanisms, we are investigating transient formation at various temperatures and attempting to determine quantum yields of formation of prelumirhodopsin as a function of temperature. It is interesting to note that a relative increase of the prelumirhodopsin band intensity at low temperatures would result in the shift of the isosbestic point to 515 nm,

Fig. 2 Difference spectrum observed 60 ps after excitation. The positive ΔA band is assigned to prelumirhodopsin while the $-\Delta A$ is due to the bleached rhodopsin. The reproducibility of each point is $\pm 0.02 A$ units. $\blacktriangle$, A 1.4/2 mm; $\bullet$, A 0.8/2 mm.

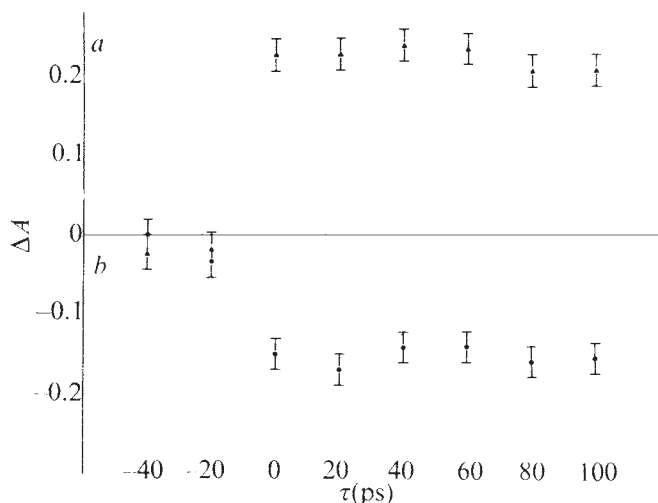
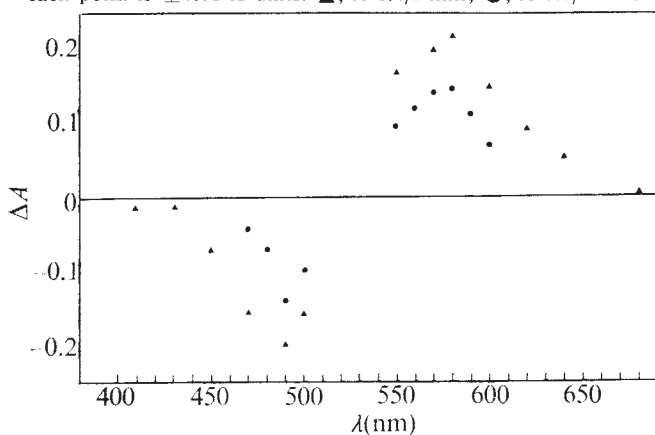


Fig. 3 *a*, Formation of the prelumirhodopsin band with a maximum at 580 nm induced by a 530-nm 6-ps pulse. $\blacktriangle$, 580 nm; $\bullet$, 480 nm. *b*, Depletion rate of the rhodopsin band at 480 nm after excitation with the same picosecond pulse. Similar kinetics are observed over the entire spectrum. Both figures represent an average of five kinetic records. The variance is given by the error bars.

which is observed at 77 K. This tends to support the above proposition that the 1 : 1 ratio observed at room temperature is probably due to active additional decay channels which deplete the excited state.

Rosenfeld and co-workers⁹ have reported the formation of isorhodopsin between 380 nm and 475 nm at 50 ns. Bensasson *et al.*⁸ reported a similar absorption occurring at 50 ns which was attributed to a new absorption due to prelumirhodopsin. As noted above, we have not observed formation of any new species between 410 nm and 430 nm between 6 ps and 100 ps nor do we have evidence for the early formation of hypsorhodopsin, the species observed by Yoshizawa¹² at liquid helium temperatures.

In view of the general agreement of our spectrum (Fig. 2) with regard to the shape, width, maxima, and kinetics (Fig. 3) with the previously published spectra for prelumirhodopsin at low temperatures, we assign the species we observe at room temperature to prelumirhodopsin. We conclude that within our time resolution of 6 ps, the only species formed at room temperature during the first 100 ps is prelumirhodopsin.

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- 1 Yoshizawa, T. & Kito, Y. *Nature* **182**, 1604–1605 (1958).
- 2 Grellman, K. H., Livingston, R. & Pratt, D. *Nature* **193**, 1258–1260 (1962).
- 3 Yoshizawa, T. & Wald, G. *Nature* **197**, 1279–1286 (1963).
- 4 Busch, G. E., Applebury, M. L., Lamola, A. A. & Rentzepis, P. M. *Proc. natn. Acad. Sci. U.S.A.* **69**, 2802–2906 (1972).
- 5 Netzel, T. L. & Rentzepis, P. M. *Chem. Phys. Lett.* **29**, 337–342 (1974).
- 6 Alfano, R. R. & Shapiro, S. L. *Phys. Rev. Lett.* **24**, 584–586 (1970).
- 7 Kaufmann, H. J. & Rentzepis, P. M. *Accs chem. Res.* **118**, 407–412 (1975).
- 8 Bensasson, R., Land, E. J. & Truscott, T. G. *Nature* **258**, 768–769 (1975).
- 9 Goldschmidt, C. R., Ottolenghi, M. & Rosenfeld, T. *Nature* **163**, 169–170 (1976).
- 10 Applebury, M. L., Zuckerman, D., Lamola, A. A. & Jovin, T. J. *Biochemistry* **13**, 3448–3458 (1974).
- 11 Tokunaga, R., Kawamura, S. & Yoshizawa, T. *Vision Res.* **16**, 633–641 (1976).
- 12 Yoshizawa, T. & Horiuchi, S. in *Biochemistry and Biophysics of Visual Pigments* (ed. Langner, H.) 69–81 (Springler, Berlin, 1973).

matters arising

Transition probability and cell-cycle initiation in yeast

SHILO *et al.*¹ have suggested that the transition probability model, devised to explain the regulation of proliferation of mammalian cells², can also be applied to yeast. They propose that traverse of an early G1 event called 'start'³ is probabilistic and is the rate-limiting step of the cell cycle. This suggestion arose from their investigation of the kinetics of bud emergence in cells released from a block at 'start', imposed either by using a strain conditionally defective in *cdc 25* or by using the mating hormone α factor. After release from a block at

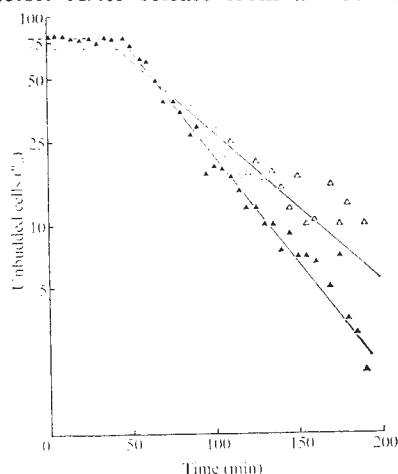


Fig. 1 The kinetics of bud emergence according to Fig. 1 in ref. 1, replotted. The proportion of unbudded cells (ordinate) is plotted on a logarithmic scale against time after release of the block (abscissa). ▲, strain 352/1 (*cdc 25*) after release of temperature block; △, same strain after release of α factor block.

'start', the proportion of unbudded cells seemed to fall exponentially, indicative of a probabilistic traverse of 'start'. Unless the proportion of unbudded cells is followed to a low level, however, it is difficult to distinguish between an exponential line and the lines derived from other distributions of cell generation times, such as reciprocal normal (A. E. Wheals, personal communication). Of the six semi-logarithmic plots given by Shilo *et al.*¹ only one continues below a level of 10% of the starting value, and in this case (Fig. 1 in ref. 1) the line curves gradually downwards. There is also scatter of the points around the fitted exponential lines, particularly in Figs 2 and 4. Therefore the evidence in

favour of a probabilistic traverse of 'start' is not convincing.

Shilo *et al.* also suggest that the kinetics of bud emergence after release from a block at 'start' are the same as in a steady state culture, indicating that 'start' is the rate-limiting step in the cell cycle. This interpretation is only possible if the kinetics of bud emergence after release from the block are due solely to the traverse of 'start' and not to physiological recovery from the block. In the examples given this is not in fact so: although it is claimed that bud emergence shows similar first-order kinetics whether the block is imposed by α factor or by *cdc 25*, this only seems to be so because the data are on linear plots. If the data are replotted semi-logarithmically (Fig. 1), the lines can be seen to be different, the half life of the unbudded state for cells after release from the α factor block being 42 min, whereas after release from the *cdc 25* block, the half-life is 29.5 min. Corresponding transition probabilities (*P* values—see ref. 2 for definition) are 0.63 and 0.76 h⁻¹. Clearly the nature of the block at 'start' is affecting the kinetics of bud emergence, which nullifies any comparison with a steady state culture.

Interpretation of these experiments is further complicated by the use of *cdc 25*. Mutants in this gene are unusual as they stop growth at the restrictive temperature, unlike mutants in most other *cdc* genes⁴. For this reason Johnston *et al.*⁵ consider it likely that *cdc 25* is affected in intermediary or macromolecular metabolism and that its arrest before 'start' is only a secondary pleiotropic effect. If this is so then the experiment plotted in Fig. 2 only determines the kinetics of recovery from this block in metabolism and is not informative about the rate limiting step of the cell cycle.

Note added in proof: Variability in event traverse is not evidence for its rate-limiting nature unless it can be established that event traverse is probabilistic. Thus, knowledge of the exact kinetics of cycle initiation (see reply by Shilo *et al.*) is essential for determining whether 'start' is the rate-limiting step of the yeast cell cycle.

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² Smith, J. A. & Martin, L. *Proc. natn. Acad. Sci. U.S.A.* **70**, 1263–1267 (1973).

³ Hartwell, L. H. *Bact. Rev.* **38**, 164–198 (1974).

⁴ Hartwell, L. H., Mortimer, R. K., Culotti, J. & Culotti, M. *Genetics* **74**, 267–286 (1973).

⁵ Johnston, G. C., Pringle, J. R. & Hartwell, L. H. *Expl Cell Res.* **105**, 79–98 (1977).

SHILO *et al.*¹ claim that their data support the concept of transition probability² in the yeast cell-cycle. However, evidence that fails to distinguish between two alternative hypotheses cannot be regarded as evidence in support of one of them. There are at least two contrasting models to account for the transition from an expandable part of the cell-cycle to a determined phase. The transition probability hypothesis argues that it occurs at random, there being a constant probability per unit time that a cell will undergo the transition^{2,3}. The cell size hypothesis suggests that cells monitor their own size and as they grow they undergo the transition when they have reached a critical cell size⁴.

Rate of entry into the budded phase is reported as showing first-order kinetics⁵, but the measurements of the fraction of unbudded cells were not continued below 2%. Above these values there is virtually no measurable difference between first-order kinetics (α -curve), a lognormal distribution⁶ and a reciprocal normal distribution⁶ as can most clearly be seen when the same data are plotted equally successfully in quite different ways, for example HeLa cells^{2,7,8} and *Euglena*^{2,9,10}. Whereas first-order kinetics is consistent with the transition probability hypothesis, both lognormal and reciprocal normal distributions are consistent with critical cell-size hypotheses and the rate of entry could be interpreted as reflecting the heterogeneity in the size of the unbudded cells and the variation in time needed to grow to a critical cell size. Concomitant measurements of size were not made. Where this has been done, attainment of a critical cell size has been shown to be an important factor in the initiation of division in, for example, *Physarum polycephalum*¹¹, *Stentor*¹², *Amoeba*¹³, *Schizosaccharomyces pombe*¹⁴, and *Saccharomyces cerevisiae* (refs 15,16) and Wheals's unpublished data.

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- ² Smith, J. A. & Martin, L. *Proc. natn. Acad. Sci. U.S.A.* **70**, 1263–1267 (1973).
- ³ Minor, P. D. & Smith, J. A. *Nature* **248**, 241–243 (1974).
- ⁴ Fantes, P. A., Grant, W. D., Pritchard, R. H., Sudbery, P. E. & Wheals, A. E. *J. theor. Biol.* **50**, 213–244 (1975).
- ⁵ Schmid, P. *Expl Cell Res.* **45**, 471–486 (1967).
- ⁶ Kubitschek, H. E. *Cell Tissue Kinet.* **4**, 113–122 (1971).
- ⁷ Hsu, T. C. *Tex. Rep. Biol. Med.* **18**, 31–33 (1960).
- ⁸ Kubitschek, H. E. *Expl Cell Res.* **26**, 439–450 (1962).
- ⁹ Cook, J. R. & Cook, B. *Expl Cell Res.* **28**, 524–530 (1962).
- ¹⁰ Kubitschek, H. E. *Nature* **209**, 1039–1040 (1966).
- ¹¹ Sudbery, P. E. & Grant, W. D. *Expl Cell Res.* **95**, 405–415 (1975).
- ¹² Tartar, V. J. *Protozool.* **10**, 445–461 (1963).
- ¹³ Prescott, D. M. in *Rhythmic and Synthetic Processes in Growth* (ed. Rudnick, D.) 59–74 (Princeton University Press, Princeton, 1957).
- ¹⁴ Fantes, P. A. *J. Cell Sci.* **24**, 51–67 (1977).
- ¹⁵ Hartwell, L. H., Culotti, J., Pringle, J. R. & Reid, B. J. *Science* **183**, 46–51 (1974).
- ¹⁶ Hayashibe, M., Sando, N., Abe, N. *J. gen. app. Microbiol.* **19**, 287–303 (1973).

B. SHILO, V. SHILO and SIMCHEN reply —We believe that the asynchronous kinetics of cell-cycle initiation are a genuine characteristic of the 'start' process¹ and not an artifact of the methods used to arrest the cells². As shown in Table 1, five different haploid strains were arrested before 'start' by a variety of methods, including temperature blocks of the temperature-sensitive 'start' mutations *cdc 25*, *cdc 28* (ref. 3), *cdc 35* (L. H. Hartwell, personal communication) and *tra 3* (ref. 4), α -hormone arrest of *a* cells, and stationary phase arrest of cells at a high titre (4×10^8 cells per ml). In some of the treatments, cells were arrested for different lengths of time. In all cases, the half life ($t_{1/2}$) of the unbudded state after release from the block was in the range of 42 ± 13 min.

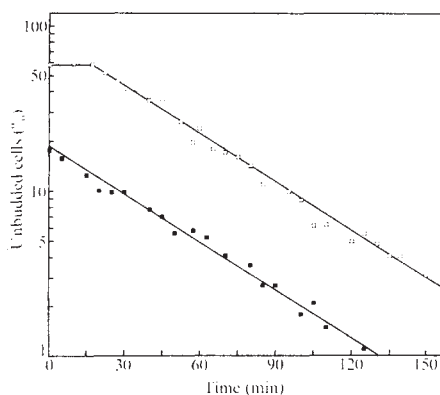


Fig. 1 Kinetics of bud initiation in an exponentially growing culture and following release from α -hormone block. The haploid strain A364A (ref. 3) is of mating type *a* and contains the markers *ade1*, *ade2*, *ura1*, *gal1-4*, *his7-1*, *lys2-2* and *tyr1-2*. The cells were exponentially grown in YEPD medium¹ at 25 °C. They were slightly sonicated¹, diluted to 1.7×10^8 cells per ml and plated on YEPD medium solidified with 2% agar, at 25 °C. After plating, the % of unbudded cells in each sample was determined by counting at least 400 cells under the microscope. In the second experiment A364A cells were plated on YEPD solid medium following release from a 2.5-h block by α mating hormone¹. ●, Exponentially growing A364A cells after plating. □, A364A cells following release from α -hormone block.

Table 1 Rates of release from blocks at 'start' and at a later stage (*cdc24*)

Strain	Mutation	Block type	Block period (h)	t_1 (min)
A364A	wild type	α -hormone	2.3, 2.5	32, 31
		Stationary	4, 4.8×10^8 cells per ml	42, 52
352/1	<i>cdc25</i>	36 °C	2, 3.3, 3.5	55, 43, 29
		36 °C	6	41
343	<i>cdc28</i>	α -hormone	2.5	39, 42, 50
		38 °C	3.8	41
353	<i>cdc 35</i>	α -hormone	2.5	38, 43
358	<i>tra3</i>	36 °C	3.3, 3.5	53, 38
337	<i>cdc24</i>	36 °C	2.8, 3, 3.3, 3.3, 4, 4.5	10, 7, 10, 4, 9, 9

All cultures were grown in YEPD medium¹ at 25 °C prior to arrest and after release from the block. Each $t_{1/2}$ value was obtained in a different experiment.

This value is five times larger than the mean $t_{1/2}$ value obtained following release from a *cdc 24* (ref. 3) block, which comes after 'start'. The variation in $t_{1/2}$ values indicates that the physiological state of the cells, the block itself and the genetic background of the strain may have some effect upon the kinetics of cell-cycle initiation. But, these effects seem only to modify, not to generate, the asynchrony in cycle initiation. In extreme cases these effects result in physiologically deprived, or even non-viable cells, which remain unbudded for a very long time. We therefore feel that data on the initiation of the last 2% of the cells would not give reliable data on the kinetics of the entire population, as has been suggested^{2,3}.

To avoid the effects of the method of arrest on cell-cycle initiation, we have conducted an experiment in which budding was monitored in an exponentially growing culture of wild-type cells. It is known that a fraction of the unbudded cells in a growing culture is located between cytokinesis and 'start'. According to the "transition probability" hypothesis⁴ this fraction is expected to decline exponentially on plating on solid medium. This was indeed observed (Fig. 1), with the frequency of unbudded cells going down from 18% to 1%, according to first-order kinetics. Moreover, the rate-constant was very similar to that obtained with the same strain following removal of α -hormone block (Fig. 1). This experiment strongly supports the suggestion that first-order kinetics are a genuine characteristic of cell-cycle initiation.

The suggestion that *cdc 25* cells are arrested at 'start' as a secondary effect of a pleiotropic mutation² is based only on the finding that they do not show any cellular growth at the restrictive temperature⁷. The latter observation can be interpreted differently, however. If the resting stage is just before 'start', we would expect cells to maintain a balanced physiological state as well as a constant cell size when arrested at 'start'. Following this rationale, the *cdc 25* temperature block

is at the natural resting stage of the cell. Even if our interpretation of the *cdc 25* mutation is not accepted, it should be pointed out that the temperature-shift experiment (ref. 1, Fig. 2) was repeated with strains carrying mutations in either *cdc 28* or *tra 3*, that were arrested at the restrictive temperature, and with *cdc 25* cells that were arrested by α -hormone. Similar results were obtained in all experiments.

It has also been suggested that the asynchronous rate of cycle initiation results from different periods required by cells in the culture in order to reach a critical size⁵. The "transition probability" model⁶ and the "critical cell size" hypothesis⁵ are not mutually exclusive, however. We have mentioned¹ the possibility that the physiological condition of the cell could affect 'start' by imposing threshold requirements such as minimal size. Indeed, a recent report⁷ presents evidence suggesting that *Saccharomyces cerevisiae* cells must reach a minimal size in order to initiate the cycle at 'start'. We suggest, however, that heterogeneity in population size alone cannot be the reason for the asynchronous rate of cell-cycle initiation. After a cell reaches the critical size, initiation of the cycle is not an immediate consequence, but seems to depend on a random event. This suggestion is supported by the following experimental findings. (1) On release from a temperature block, the first buddings of the mutant *cdc 24* on solid medium appear synchronously (ref. 1, Fig. 1). The second buddings are asynchronous, however, following similar kinetics to that of cell-cycle initiation with a $t_{1/2}$ value of 31 min. The theory of critical size cannot explain asynchronous budding of cells which have already produced at least one bud (and apparently reached the critical size). (2) 'Start' arrest of *cdc 28* cells at the restrictive temperature, or *a* cells arrest by α -hormone, do not block cell growth, in contrast to *cdc 25* temperature arrest⁷. Similar kinetics of cycle initiation were observed in all three cases, however (Table 1).

In conclusion, we feel that the experimental data given here and in our previous publication¹ support the "transition probability" model⁶ for cell-cycle initiation, though admittedly additional variables may have to be taken into account. Discussions about the exact kinetics of cycle initiation, however, should not obliterate our main conclusion about 'start', namely that it is the rate-limiting step of the yeast cell cycle.

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- ¹ Shilo, B., Shilo, V. & Simchen, G. *Nature* **264**, 767-770 (1976).
- ² Nurse, P. & Fantes, P. *Nature* **267**, 647 (1977).
- ³ Hartwell, L. H., Mortimer, J., Culotti J. & Culotti, M. *Genetics* **74**, 267-286 (1973).
- ⁴ Wolfner, M., Yep, D., Messenguy, F. & Fink, G. R. *J. molec. Biol.* **96**, 273-290 (1975).
- ⁵ Wheals, A. E. *Nature* **267**, 647-648 (1977).
- ⁶ Smith, J. A. & Martin, L. *Proc. natn. Acad. Sci. U.S.A.* **70**, 1263-1267 (1973).
- ⁷ Johnston, G. C., Pringle, J. R. & Hartwell, L. H. *Exp Cell Res.* **105**, 79-98 (1977).

Fission-track dating of pumice from the KBS Tuff, East Rudolf, Kenya

HURFORD, Gleadow and Naeser¹ claim to have fission-track dating results supporting the controversial 2.61-Myr value for the age of the KBS Tuff in East Rudolph, Kenya as determined by K-Ar dating²⁻⁴. The fission-track age does not, however, contribute substantially to solving this controversy, particularly since the authors¹ have not drawn attention to two important points, namely the error limits of the age and the current uncertainty about the spontaneous fission constant of uranium-238.

First, the quoted error of about 3% seems unrealistically small and probably represents only precision. The authors should give also the age accuracy which is necessary for comparing different radiometric ages. Second, many fission-track specialists no longer use the $6.85 \times 10^{-17} \text{ yr}^{-1}$ value, but now use as the decay constant $8.46 \times 10^{-17} \text{ yr}^{-1}$; there are good reasons for this preference⁵. If this higher value for the decay constant is used, the fission-track age of the pumice in the KBS tuff recalculates to 1.98 Myr, which would lend support to the K-Ar age measured by Curtis *et al.*⁵. For stratigraphic use of fission-track ages one has to be critically aware of, and draw attention to, the present uncertainty of the spontaneous fission constant of uranium-238.

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- ¹ Hurford, A. J., Gleadow, A. J. W. & Naeser, C. W., *Nature* **263**, 738-740 (1976).
- ² Fitch, F. J., Findlater, J. C., Watkins, R. T. & Miller, J. A. *Nature* **251**, 213-215 (1974).
- ³ Curtis, G. H., Drake, R. F., Cerling, T. E., Cerling, B. L. & Hampel, J. H. *Nature* **258**, 395-398 (1975).
- ⁴ Fitch, F. J., Hooker, P. J. & Miller, J. A., *Nature* **263**, 740-744 (1976).
- ⁵ Wagner, G. A. *et al. Geochim. cosmochim. Acta* **39**, 1279 (1975).

NAESER, HURFORD AND GLEADOW
REPLY—We feel that the age we reported is a reasonable estimate for the age of the zircons separated from the pumice lumps in the KBS Tuff. If our age is wrong, it is wrong for reasons other than our choice of the decay constant² for the spontaneous fission of ²³⁸U. Two possible sources of error in this age are geologic in origin:

(1) The samples were collected in a sedimentary sequence. In this type of occurrence, contamination by detrital zircons is always possible and, in fact, is quite common. One advantage of the fission-track dating method is that the age of single crystals can be determined. A detrital zircon having an age greater than 10 Myr can easily be excluded from the population being dated. The problem occurs when the contaminating zircons are only a little older than the zircons being dated. The statistics of individual grains are such that a zircon having an age of 6 Myr would be included in the data because it cannot be reasonably separated from the rest of the population. In this case, however, five different determinations were made by three different individuals, and it seems highly unlikely that all three would choose the same relative numbers of contaminating grains. For this to happen, the detrital and pyrogenic zircons would have to be present in equal proportions, and the age of the detrital zircons could not be much greater than about 3 Myr.

(2) These zircons contained many small needle-like inclusions. Some of these could possibly have been counted as tracks, and this would result in an older age. As was true for the first source of error, this type of counting error would have to have been made by all three laboratories to the same extent.

Wagner² has questioned our choice of a decay constant, $\lambda_F = 6.85 \times 10^{-17} \text{ yr}^{-1}$ (ref. 3). When it is used in conjunction with the fission track glass standards of the U.S. National Bureau of Standards⁴, we get the best agreement with the K-Ar ages of co-existing minerals and we use it for this reason. This agreement has been found for minerals such as zircon, apatite, and sphene, as well as natural glasses that have not suffered track annealing. Figure 1 shows the results of 34 zircon fission-track ages plotted against the average K-Ar age of one or more minerals from the same rock. These ages are all from volcanic or sub-

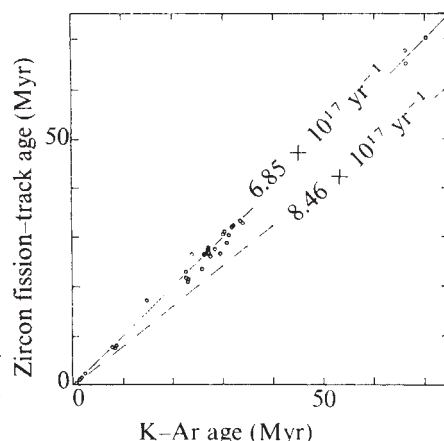


Fig. 1 Zircon fission-track ages and the average K-Ar ages of minerals from volcanic and subvolcanic rocks. λ_F values $6.85 \times 10^{-17} \text{ yr}^{-1}$ (solid line) and $8.46 \times 10^{-17} \text{ yr}^{-1}$ (broken line).

volcanic rocks in which annealing should be absent or minimal. Alternatively we could have chosen an empirical method⁵ to calculate the ages of the KBS Tuff zircons. This method is independent of λ_F and neutron-dose calibration, simply requiring a number of samples from well-dated rocks. Had we chosen this method, our results on the zircons from the KBS Tuff would have been the same.

Wagner has also questioned our statistics². The precision of a single fission-track age determination is not equal to that of a K-Ar age, and probably never will be, but five separate determinations can produce a mean that has a reasonably small error associated with it. The accuracy of any age can only be guessed at, in that we do not know the true age of any geologic sample. We can only strive for the best agreement with K-Ar and the other dating methods.

We therefore think that our age of 2.4 Myr is a reasonable estimate of the age of zircons separated from the pumice lumps present in the KBS Tuff. If Wagner feels that we must use $\lambda_F = 8.4 \times 10^{-17} \text{ yr}^{-1}$, then he must show us where we have a corresponding 20% error in our method that compensates for our choice of decay constant and that will account for our close agreement with K-Ar ages.

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- ¹ Hurford, A. J., Gleadow, A. J. W. & Naeser, C. W., *Nature* **263**, 738-740 (1976).
- ² Wagner, G. A., *Nature* **267**, 649 (1977).
- ³ Fleischer, R. L. & Price, P. B. *Phys. Rev.* **133**, 1363 (1964).
- ⁴ Carpenter, B. S. & Reimer, G. M., *NBS Sp. Pub.* **260-49** (1974).
- ⁵ Fleischer, R. L., Price, P. B. & Walker, R. M., *Nuclear Tracks in Solids* (University of California Press, Berkeley, 1975).

Southern oscillation index and atmospheric carbon dioxide

BACASTOW¹ claims that the Southern Oscillation (SO) modulates atmospheric carbon dioxide, which may be true, but was not proven by his article.

Bacastow compared a SO index with both carbon dioxide concentrations and its time derivative. In his Fig. 1 carbon dioxide concentrations were smoothed in an unspecified manner. Taking the derivative is equivalent to applying a high pass filter to the data and results therefore depend on the original smoothing. With different forms of smoothing applied to each series the phase relations will be affected.

The period of data used in the study was from 1958 to 1974, which includes the period 1960–1965 when the SO was not operating effectively. Trenberth² shows that, in the early 1960s, the correlations of pressure between Easter Island and Darwin—required by the SO mechanism to be negative—were in fact positive. He further shows that the SO operates only on a time scale of 2–10 yr and mainly in the 3–6-yr range. Marked quasi-biennial fluctuations occur at Darwin, but not Apia, Tahiti, or Easter Island in the South Pacific. The quasi-biennial oscillation and the SO are not apparently the same thing, although they may interact at times. Furthermore, lags of up to 1 yr are involved in the opposite changes at Darwin and Easter Island³.

Further consideration should therefore be given to the choice of a SO index. Trenberth suggests that a simple index involving only Darwin and Tahiti would

Table 1 Southern Oscillation index correlation with CO₂ data

Derivative anomaly	SO index	Smoothing	Correlation maximum	Anomaly lag (month)	Standard deviation
South Pole	$P_{EAS} - P_{DAR}$	12 month running mean	-0.64	6	0.34
Mauna Loa	$P_{EAS} - P_{DAR}$	12 month running mean	-0.55	2½	0.28
South Pole	$P_{UAS} - P_{DAR}$	Spline	-0.70	6	0.36
Mauna Loa	$P_{EAS} - P_{DAR}$	Spline	-0.56	2½	0.31
South Pole	SO1	3 month running mean	-0.60	4	0.26
South Pole	SO2	3 month running mean	-0.59	4	0.24

P_{EAS} and P_{DAR} , seasonal pressure anomalies at Easter Island and Darwin respectively.

be best since these stations have the highest negative correlation (-0.76 for yearly values for 1944–73) and are almost exactly out of phase. This could be used to define lag relationships elsewhere. Seasonal, rather than monthly data should be used.

To find such an index, we note that an exact inverse correlation between Darwin and Tahiti, using their standard deviations gives

$$P_{\text{Darwin}} = -1.26 P_{\text{Tahiti}} \quad (1)$$

where P is the seasonal pressure anomaly. Since part of the mass compensation from the Indonesian centre of action of the SO occurs south of Australia and New Zealand as well as in the South Pacific³, greater weight should be given to the pressure at Darwin in a SO index (see Fig. 6 of ref. 3). A suitable weighted index, based on covariances, is

$$\text{SO1} = P_{\text{Tahiti}} - 1.2 P_{\text{Darwin}} \quad (2)$$

As only two stations are included, some noise would be present, but is measured by

$$\text{SON} = 1.26 P_{\text{Tahiti}} + P_{\text{Darwin}} \quad (3)$$

which from (1), has a zero value if the SO is operating.

An index that combines (2) and (3) is

$$\text{SO2} = \frac{P_{\text{Tahiti}} - 1.2 P_{\text{Darwin}}}{1 + |\text{SON}|} \quad (4)$$

I suggest that this index may be more useful than any previously used.

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¹ Bacastow R. B. *Nature* 261, 116–118 (1976).

² Trenberth, K. E. *Q. Jl R. met. Soc.* 102, 499–513 (1976).

³ Kidson, J. W. *Mon. Wea. Rev.* 103, 187–196 (1975).

BACASTOW REPLIES—Trenberth is correct in pointing out that different filtering of two time series will, in general, affect their correlation-time lag relationship. I do not believe, however, that this consideration is important in judging the existence of a connection between the Southern Oscillation (SO) and the atmospheric CO₂ level. If a Southern Oscillation Index is generated by smoothing the Easter Island–Darwin, Australia, pressure differences by the procedure applied to the CO₂ data, the cross correlation functions of this index with the time derivatives of the CO₂ anomalies (see Table 1 for summary) are essentially the same as found for these pressure differences smoothed by a 12 month running mean.

The mechanism behind the SO is poorly understood, so the best index to represent it is unclear. The indices suggested by Trenberth (calculated from data in ref. 1) are qualitatively quite similar to the Easter Island–Darwin Index, except for the obvious difference in degree of smoothing (Fig. 1). Pressure anomalies were smoothed by a 3-month running mean, in accordance with Trenberth's suggestion that seasonal data be used. Cross correlation functions of SO1 and SO2 with the CO₂ anomaly derivative at the South Pole (Table 1) are very similar to those found for the Easter Island–Darwin Index, except that the lag of the anomaly is about 2 months less relative to the Tahiti–Darwin indices, as would be expected from the analysis of Trenberth².

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² Trenberth, K. E. *Q. Jl R. met. Soc.* 102, 499–513 (1976).

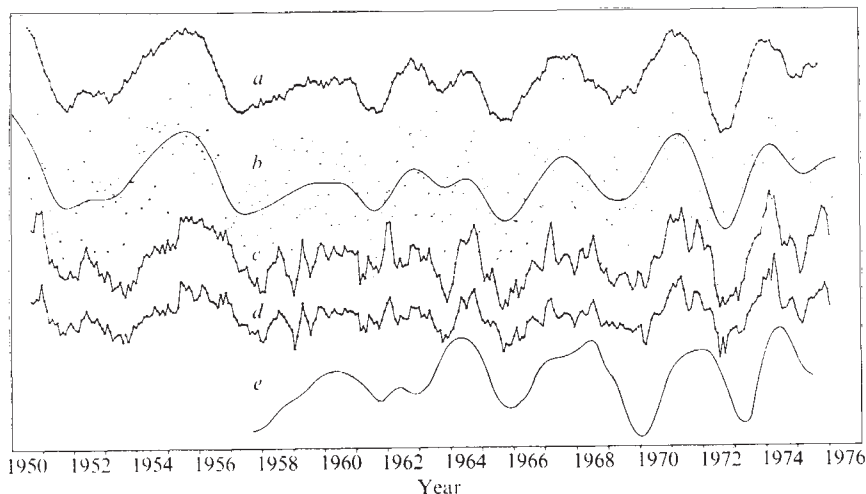


Fig. 1 Southern Oscillation indices. *a*, Differences in monthly average pressure between Easter Island and Darwin, Australia, smoothed by a 12-month running mean (which also removes the seasonal effect). *b*, Pressure differences above with the seasonal effect removed. The curve is a spline obtained by the procedure used to find the CO₂ anomalies, and, accordingly, the stiffness of the spline was adjusted by eye. The seasonal effects were calculated by an average over identical months of the difference between the pressures and the spline. *c*, SO1. The seasonal effects at Tahiti and Darwin were determined by an average over identical months of the monthly average pressure records (since 1950) and removed from each record by subtraction. Each record was then smoothed by a 3-month running mean and SO1 calculated. *d*, SO2. *e*, Inverted time derivative of the South Pole anomaly, for comparison.

reviews

Disaster book

Peter J. Smith

Forces of Nature. Edited by Sir Vivian Fuchs. Pp. 303. (Thames and Hudson: London, 1977.) £9.50.

PERHAPS UNWITTINGLY, this book plays a small part in a curious paradox. For the past few years the world economy has been in recession, human institutions have been under threat as seldom before, and the general feeling has been one of gloom and despondency. But as if that were not enough, the same period has seen a remarkable upsurge in the publication of books about those macroscopic forces of nature which can, and do, contribute to recession, destroy human institutions and give rise to gloom, not to mention real hardship. The level of interest in earthquakes, volcanic eruptions, drought, floods, avalanches, tornadoes and forthcoming ice ages has recently reached a new height and shows no sign of falling. It is almost as if we were trying subconsciously to diminish the relative force of human mismanagement by pointing to greater events beyond our control.

But if so, we are doomed to failure, for what Sir Vivian's book tells us time and again is that in the face of disruptive natural forces human folly is only magnified. The ancient city of Antioch was repeatedly destroyed by earthquakes and equally repeatedly rebuilt on the same site with similar materials and no more effective rules—a self-defeating activity which even the most highly developed nations have been unable to avoid. In 1963 the Vaiont reservoir in Italy overflowed violently with the loss of 2,600 lives, because no-one had read the lessons plainly visible in the local history of landslides. In Switzerland the obvious dangers of avalanche are often ignored even by experienced mountaineers. In countries where drought is endemic, populations grow unchecked, making each disaster more devastating than the last. And so on.

Under the circumstances it is perhaps understandable that Sir Vivian should take a dim view of human capability. Of all the forces of nature, he suggests in his introduction, "none can be controlled by human agency. Even if our growing knowledge was to make this possible, it is doubtful if we have, or could acquire, the wisdom to handle

them for the general good . . . It may well be wiser to accept the broad interplay of natural forces, always learning better how to use them or to protect ourselves from them." Not everyone would agree with that, regarding it as overly defeatist, for the reference to protection reminds us that man is not entirely incapable of fighting nature. From the Tennessee Valley Authority (for all its faults), through splitting wedges in the Alps and the sea wall at Skegness, to the person who decides that Denver offers a safer life than Los Angeles, there is ample evidence that nature needed not always win.

In *Forces of Nature*, each chapter of which is written by "an expert in his field", man's attitudes and responses to natural phenomena are given more than usual attention, although firmly set against a background of scientific explanation. The blend of science and sociology works well, leading to a nicely balanced text that should have wide appeal. What ultimately makes the book, however, is less the text than the remarkable collec-

tion of photographs (researched by Sarah Waters), some in black-and-white but many in colour.

And therein lies something slightly unnerving. For the truth is that in the hands of a brilliant photographer, and with a magnificent standard of reproduction, the consequences of the most devastating natural disaster may be recorded as something of astonishing beauty. The funnel of a tornado crossing Oklahoma, a parched and drought-cracked river bed in Kenya, rooftops projecting from a major flood near Brisbane, the distortion of a flood-swept railway line in the Netherlands, and molten lava from Etna advancing towards civilisation, must all have represented absolute despair to someone; and yet on the page they make pictures as enticing in their way as a *Hay Wain* or a *Fighting Temeraire*. It's all vaguely disconcerting, but makes for a fascinating book. □

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Time problems in land processes

Geomorphology and Time. By J. B. Thornes and D. Brunsden. Pp. 208. (Methuen: London, 1977.) Hardback £6; paperback £3.45.

WHAT George Poulett Scrope wrote in 1858, that "the leading idea which is present in all our researches, and which accompanies every fresh observation, the sound which to the ear of the student of Nature seems continually echoed from every part of her works, is—Time! . . . Time! . . . Time!", could be equally well be applied to studies today. It was the geologist who discredited the rigid biblical timescale and extended the overall limits of earthly time from under 6,000 years to over 4,000 Myr. Geomorphologists joined the geological bandwagon with one hand on the leading rein. Quite early they began to be interested in rates of processes, and eventually acquired an assortment of morphological measurements and crude timescales

for the effects of denudation and deposition on landforms.

Being concerned with present processes, geomorphology has contemporary utility, and the hope has always been that the present would also provide a key to the past. This is of course, true of the action of processes and untrue of landscapes. In any event, time-independent processes cannot exist. So time is a necessary ingredient of geomorphological research and must be considered with other essential features such as lithology, process and spatial variability, all of which impinge on problems of scientific observation, model-building and interpretations of the temporal occurrence of events.

The authors of this book elucidate these ideas on time in a lively introduction which treads warily around many pitfalls and will stimulate much thought. For example, we are told that velocity is expressed as distance moved in unit time (L/T). This seems reason-

ably applicable to large deep rivers, but I wonder if, in fact, in steep shallow channels with rocky, pot-holed beds, where some water movement is uphill, geomorphologists do measure the true distance travelled by a water particle in a given length between two successive points. Among the many interesting topics mentioned in these introductory pages are relaxation time and feedback in energy systems.

The following chapter discusses at some length the criteria that Earth scientists use to obtain a chronology. Relative morphological vertical position, extent of drainage patterns, stratigraphy, mineral and organic content, weathering, artefacts, radiation, tree rings and lichenometry all find a welcome place, but thermoluminescence might perhaps have been better replaced by palaeomagnetism. The discussion then proceeds to the measurement of variables in time, the analysis of temporal data, and the rates of operation of geomorphological processes. In these the authors rightly consider that awareness of techniques will make the reader more careful in designing experiments for the collection of time-based data and more aware of the inadequate nature of much of the published data on measured rates of processes.

The next three chapters of the text provide information on the various types of models concerned with the way that events change in time. The qualitative temporal variety leads on to the quantitative deterministic type and so to the less certain stochastic models of form evolution. Not surprisingly, as geomorphologists deal with area realities, there follows a discussion on the setting of events or points into a framework wherein spatial characteristics or patterns evolve. A summary of space, and time and a long bibliography of mainly post-1960 literature, form a suitable conclusion.

In brief, this neat and well-illustrated book provides at a reasonable price a commendable introduction to recent conceptual advances in time-related factors in geomorphology. Geomorphologists may have far to go and some time to wait before their recordings on rates of process become truly reliable but this excellent summary will provide students with a clear view of the time-scale aspect of present achievements.

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Collagen biochemistry and biophysics

Biochemistry of Collagen. Edited by G. N. Ramachandram and A. H. Reddi. Pp. xv+536. (Plenum: New York and London, 1976.) \$59.40.

UNTIL some 30 years ago, collagen was of interest mainly to industrial chemists concerned with the properties of leather, glue and gelatine. Since then, there has been a rapid expansion of information concerning collagen structure, metabolism and function. This book, as the title implies, covers the biochemistry of collagen but also contains chapters more properly labelled biophysics.

The chapter by Piez collates the information available on the primary structure of collagen polypeptide chains. Also discussed are the sequences involved in the unique post-translational modifications, particularly hydroxylation and crosslinking. There are brief descriptions of the methods of isolation of the different collagens and of the polypeptide fragments to which the usual methods of sequencing have been applied.

The chapter by Ramachandran and Ramakrishnan provides a general account of the early X-ray diffraction studies which led to the formulation of a triple-helical structure for collagen, together with a more detailed consideration of recent modifications of this basic structure. The chapter on synthetic polypeptide models is largely redundant, much of the material being covered by other authors.

The molecular packing of collagen monomers in fibrils is the subject of a chapter by Miller who discusses critically the methods available for studying this packing, particularly X-ray diffraction and electron-microscopy, and the results obtained by these techniques. More tentatively, he discusses the possible models which can be derived from the data available.

The complex processes by which the collagen molecule is synthesised are dealt with by Prockop and his colleagues. After a short section on collagen mRNA, the manifold post-translational modifications which the primary structure of collagen undergoes are discussed. These include hydroxylation of lysine and proline, and glycosylation of hydroxylysine. Also discussed is the final intracellular process which involves the assembly of three polypeptide chains and the subsequent triple helix formation before the

molecule is secreted. The final section is a stimulating discussion of the regulation of these intracellular processes by which the wide range of chemically different collagens found in the body are achieved.

After secretion the completed molecule is cleaved to form a molecule which can form a fibril by a process of self-assembly. The stabilisation of the fibrillar structure by covalent crosslinks is discussed in a chapter by Tanzer. This deals with the structure and mechanism of formation of these crosslinks.

The enzyme mammalian collagenase, one of several proteases involved in collagen degradation, is the subject of a very useful chapter by Gross. The most interesting section is that concerned with the regulation of collagenase activity and how it is synchronised in time and space with collagen biosynthesis.

The chapter by Timpl on immunological studies is concerned with the diversity and localisation of antigenic determinants on the collagen molecule. There are useful sections on the use of specific antisera to detect anticollagen antibodies and also as tools to study the structure and metabolism of collagen. There is a section on the use of fluorescent collagen antibodies to localise and follow the temporal changes in the appearance of the different genetically determined collagen.

The molecular basis of diseases affecting collagen is dealt with in an excellent chapter by Lapière and Nugens. This contains two tables of data on the cellular and molecular basis of collagen defects, and on the techniques applied to define collagen pathology. Various diseases of connective tissue, both inherited and acquired, are discussed in the light of current knowledge of collagen structure and metabolism.

The chapter by Reddi is somewhat disappointing. It covers rather sketchily an area of research concerning the intimate involvement of collagen in cell differentiation and organogenesis. This is an important topic requiring a more detailed and critical review than that provided by this chapter.

There is evidence of a considerable gap between the completion of the manuscript and final production of the book (compensated in some chapters by addenda). Nevertheless this is a reasonably up-to-date account of the current status of collagen research which should be useful to workers in a wide variety of disciplines.

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Ecological parasitology

Ecological Aspects of Parasitology. Edited by C. R. Kennedy. Pp. x+474. (North-Holland: Amsterdam and Oxford, 1976.) Dfl 145; \$57.95.

PARASITISM is an ecological term, and parasitology is essentially an ecological study. Some aspects of parasite ecology have received extensive coverage for many years, especially those related to qualitative and quantitative epidemiology, where the unit of study is the infection rather than the parasite.

The stated object of this book is to remedy the situation whereby some aspects have received little attention. The aspects chosen in the first section concern the parasite outside the host: dispersal (C. R. Kennedy), host location (A. J. MacInnis) and host selection, (J. C. Holmes); entry into the host (D. W. T. Crompton), feeding (C. Arme), interaction between parasites (D. Halvorsen), and host responses to parasitic infection (D. Wakelin). The bulk of the book is in part two. Here, "Habitat" is seen as the immediate surroundings of the parasite. There are two chapters on fish: skin (G. C. Kearn) and gills (C. H. Fernando and C. Hanek); and the remaining chapters deal with the special features of parasitic life in different mammalian or avian organs and cells. In the final chapter, R. M. Anderson discusses the interaction of host and parasite populations.

With 24 authors covering so wide a field, consistency and uniformity are not to be expected or even desired. If the intellectual verbosity of chapter one were maintained throughout, the book would be hard indeed to read. Particularly noteworthy is yet another definition of parasitism, first credited with elegant beauty, then, mercifully, discredited as being possibly applicable to all organisms!

The rest of the book is clear and stimulating. The only truly unique feature of the parasitic habitat is the specific antagonistic reaction that it produces. This aspect is given extensive treatment. D. R. Arthur describes how amputee mice, unable to groom, are subject to massive infestations of lice; D. Wakelin gives a lucid comparison between the immune processes of invertebrates and vertebrates, and A. D. Befus and R. B. Podesta describe the special immunological features of the intestine.

Most other aspects of parasite ecology have clear parallels in free living organisms. Useful comparisons are made but unfortunately not ex-

panded with both sessile and planktonic marine animals. More such comparisons could have been enlightening.

The great value of this book lies in the descriptions of various organs from a parasitological viewpoint. Almost every author points out areas of extensive ignorance in his field, and it is clear that too few physiologists, biochemists and physicians have a clear idea of what ecology is about. The book will surely be useful as a

stimulant for discussion and further investigation. It is a little disappointing that in so extensive a series of reviews there is a sad lack of coordinating thought. Perhaps, though, that is what the book will help to produce.

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Electron theory of small molecules

Introduction to the Electron Theory of Small Molecules. By A. C. Hurley. Pp. 329. £12; \$26.25. *Electron Correlation in Small Molecules.* By A. C. Hurley. Pp. 276. £10.50; \$23. (Academic: London and New York, 1977.)

THESE two books are perhaps meant to be read together. The author states in his preface to the *Introduction* that the book consists of the "earlier and simpler chapters of what was originally conceived of as a comprehensive one-volume work". As such, the *Introduction* is to provide what the author calls a "royal road from basic quantum mechanics to the various theories which are now yielding such detailed information on molecular interactions", some of which are discussed in the *Electron Correlation* volume.

The *Introduction* provides an account of the basic theory of potential energy curves and surfaces, an outline of the variational method for constructing approximate electronic wave functions and a short account of the (generalised) virial and Hellmann-Feynmann theorems. It also provides a brief, but self-contained account of molecular symmetry and quite extended accounts of the determinantal approach and of molecular orbitals and the Hartree-Fock method. The accounts are presented chiefly in the context of results for diatomic molecules (although small polyatomics occasionally receive a passing mention) and a chapter each is devoted to detailed consideration of the hydrogen ion and of the hydrogen molecule.

The book is regrettably something of a disappointment to the reviewer, if for no other reason than that it seems so old-fashioned. A suspicion of this may be gleaned from the first few pages in which it becomes clear that the author is going to use c.g.s. units; but it is re-inforced, for example, by his treatment of spin-coupling and of methods for solving the SCF equations,

for which he gives no references more recent than 1967. Indeed, if one looks through the text one gains the impression that the phrase "recent work" should really be synonymous with "ten-year-old work". This criticism should not, of course, be construed as a call for "trendiness", but there is work in the past five years in the areas quoted above (and indeed in other areas in the book) to which the attention of the reader should be called even in an introductory book like this.

As one would expect from an author of Dr Hurley's distinction, there are a lot of good things in the *Introduction*. There is, for example, a splendid and precise account of Koopmans' theorem, a very nice heuristic account of the various cusp conditions in the wave function, some very helpful material on the continuous groups in the section on symmetry, a brief and clear account of the RKR method, and many more. There are, however, some funny slips. The author says, for example, that in the quadratic approximation to the potential, the equation for nuclear vibrational motion in the diatomic is the simple harmonic oscillator equation. But the student who has read his Eyring, Walter and Kimball *Quantum Chemistry* knows that this is just not true. The equation (2.60) from which he begins his account of the generalised Hellmann-Feynmann theorem seems to be simply wrong at an elementary level (see the author's own original paper, *Proc. R. Soc. A* **226**, 170 or Appendix 1 in Pople *et al.* *Jl Chem. Phys.* **49**, 2960; 1968), and in view of Dr Hurley's contributions to the foundations of this part of the subject this (if the reviewer is not mistaken) is quite incredible. Be that as it may, the discussion in this part of the book has nothing like the subtlety or power of the discussion of the same material offered by Epstein in his recent book (*The Variation Method in Quantum Chemistry*, Academic: London, 1974); and Epstein's book is not referred to at all.

At a somewhat different level, the author's discussion of natural orbitals

(continued on p654)

will strike the initiated reader as very eccentric; and I do not think it will help the beginner. The index of the book also seems to have its eccentricities. If one looks up "charge density" for example, one gets referred to two pages on neither of which is the concept defined or anything other than incidentally mentioned. One does not get referred to the section on p303, which has charge density in its heading, which is perhaps just as well because the section contains no mention at all of the idea.

The book cannot therefore be recommended unreservedly as an introduction to the field but it is nevertheless a book that repays careful reading and should be useful to those teaching advanced undergraduate and graduates in the subject, if only because there is not really another book in this area.

Electron Correlation consists of just two chapters, one entitled "First Order CI and VB methods" and the other (about 200 pages) "The Correlation Problem". Again, the emphasis is on diatomics but polyatomics get a better look in than in the *Introduction*. The underlying and unifying theme of the book seems to be designing wave functions for molecules which correctly describe their dissociation into fragments. In the first chapter the author takes a rather eclectic view of CI and VB, and the reader looking for detailed accounts of the current state of the art in these methods will be disappointed. In fact most of the chapter is devoted to a discussion of symmetry principles, of a particular multi-configuration SCF method and of an introduction to the theory of separated electron pairs. The second chapter is devoted almost

entirely to pair theories culminating in an account of the coupled pair approximation treated in a manner equivalent to Cizek's treatment. After a short discussion of the requirements necessary to obtain chemical accuracy using such methods the chapter ends with a brief account of putting r_{12} explicitly into the wave function particularly by the *trans*-correlated method.

Once more there are lots of good things in this book. There is a splendid account of natural spin-orbitals and the convergence of the CI expansion, and of the difficulties in iterative pseudo-natural orbital approaches. There is a very clear and precise account of the deficiencies inherent in particular classes of wave functions constructed from strongly orthogonal groups. Indeed, the whole account of development of pair theories is clear, coherent and precise, and extremely well illustrated by results from actual calculations.

Again it does seem to lack references to more contemporary work: obvious omissions are the work of Meyer and that of Ahlrichs and his group in the pair function field. One would also expect to see more references to recent work in the CI and VB approaches. The result is that the book must strike the informed reader as a somewhat unbalanced account of the field.

The reviewer's reservations here are, however, only minor. *Electron Correlation* is a book that many practising quantum chemists will surely want to own.

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Ice Age, ancient and modern

The Ice Age: Past and Present. By Brian S. John. Pp. 254. (Collins: London, 1977.) £4.95.

ADDING to the rapidly expanding recent literature on glaciers, Ice Ages and such topics, Brian John has produced a useful non-technical volume that is designed to appeal to "the person who knows very little about ice and glaciers and even less about the Ice Age". In 235 pages it covers a great deal of ground, from how glacier ice is formed to its effects on the Earth's surface, from ancient Ice Ages to speculative comments on future climatic change. Inevitably the treatment becomes somewhat superficial in places, as when the botany of the Ice Age is surveyed in just 12 pages; but on the whole it

succeeds very well in dealing with such a huge canvas. Moreover, it is a soundly based book: on geomorphological and geological aspects, John knows his stuff, and therefore unlike many popular or semi-popular accounts, the text is reliable and up-to-date.

The arrangement of material is clear and logical. The first half of the book looks at the nature and origins of glaciers, their effects on landforms through erosion and deposition, the landscapes associated with both continental and alpine types of glaciation, and the work of glacial meltwater in washing out and transporting debris away from the edge of the ice. There follow short chapters on ice in a periglacial environment, touching briefly on permafrost, frost weathering, patterned ground, pingos and other ground-ice features, and on sea ice, dealing with ice shelves, pack ice and icebergs.

The second half of the book deals mainly with Ice Ages. Sensibly, for

this type of book, John begins with the recent historical past, discussing the effects and significance of the "Little Ice Age", before delving further into the past to consider the Pleistocene period and then the pre-Pleistocene glaciations, as well as the innumerable theories of the causes of Ice Ages. There is an outline of the effects of glaciation on land and sealevels; a minor criticism here is that *non*-glacial causes of changes in level tend to be ignored so that one gets the erroneous impression, for instance, that the sinking of the North Sea basin is somehow bound up with glaciation. Three chapters provide brief summaries of Ice Age plants, animals and man, and the book concludes with a discussion of what the future may hold in store—when will the present interglacial come to an end, how will it end, and what impact is man having on the world's glaciers, intentionally or unintentionally?

Throughout, John tries to write in a simple non-technical style. On the whole he probably succeeds in making the subject matter intelligible to the non-specialist, but often at the risk of over-simplification. At times, however, he lets his imagination run away with him. Such statements as "You don't have to ask an Eskimo whether we are living in the middle of an Ice Age", or "Like the Irish navvies of Victorian times, the most active glaciers tend to be the most efficient, and where they have plenty of excess energy they achieve prodigious feats of erosion and transportation", are probably acceptable in a book of this sort, but some other passages draw rather far-fetched similes. Did he really have to write that "Unstable glaciers cannot find relief from the stresses of life by weeping or getting drunk", or that "Pingos prefer to live in groups . . ." (they are described as "lovable creatures" because they tend to cluster together)?

The book is well produced with few errors. An amusing slip is 'subsiding' instead of 'subsiding' on p230. The 32 half-tone plates are mostly of high quality (the ERTS imagery of Skeidararsandur is an exception), and the line-drawings are clear. The latter, oddly, are not numbered and not specifically referred to in the text. There is a short list of books for further reading, and an index. At £4.95, the book represents reasonable value for money; it ought to attract the non-specialist reader and it will certainly stimulate the intelligent sixth-former (advanced school) student to find out more about ice and Ice Ages.

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